

Reagan votes for basic research

The budget deals generously with basic science; the snags are that the election will tempt Congress to deny the President what he wants and that the strategy of budget deficits may blow up in his face.

PRESIDENT Ronald Reagan's pre-election budget is what his pollsters had been expecting and will not surprise even his would-be opponents next November. Between now and then, politicians and voters alike will be less concerned with what is called the bottom line — the huge deficit yet again in prospect — than with the details of spending plans for the financial year that will have just begun by election day. In the circumstances, it is commendable that the new budget is less like an electioneering document than it might have been. Moreover, other things being equal as the economists say, the budget could do much to strengthen basic research in the United States. So the important question is whether Congress will let good things come about. And while there is no reason to suspect that Congress has its knife into basic science, the danger is high that in the run-up to the election, large chunks of the budget will be so encumbered with spending plans the administration cannot accept that the business of the United States will be managed by continuing resolutions until after the votes have been counted. Those planning to order new equipment on the strength of last week's budget will prudently contain themselves in patience.

Of all the components of the budget, the most welcome because the most overdue is the proposal that federal support for agricultural research should be put on a more rational basis. The plan is that, from next October, the US Department of Agriculture should have \$50 million to spend on research grants awarded competitively, twice as much again as in the year now under way. This shift of resources, in magnitude only modest, will be significant if it can be taken as a commitment by the administration to the principle that agricultural research in the United States should be reformed.

Opposition

Handing out large parcels of public money to the land-grant colleges was a powerful stimulus to self-conscious agriculture at the end of the nineteenth century, while the Department of Agriculture's own research establishments were fruitful sources of basic science as recently as half a century ago. But times have changed. It is now comically anomalous that agriculture, the most important single industry in the United States, should uniquely be deprived of more than token access to the groups of imaginative people in US universities willing and able to break a lance or two in the interests of the agriculture of the future — and without demanding a public service contract and pension in return. None of this is to pretend that competition for research grants, and the peer reviews that attend them, are free from blemish or to deny the value of what industrial companies are now spending — perhaps \$100 million a year — on basic research in agriculture. But it will be a test of the administration's seriousness towards research that it should be prepared to battle for the modest proposal now put forward in the face of the entrenched opposition there will be in Congress.

The next most welcome development is the proposed generosity towards the National Science Foundation, still no better endowed in real terms than in the early 1970s. Again it makes sense that the only agency of government able to support projects not tied to what are called missions (public health included) should have more funds at its disposal when executive agencies and private industry are both busily spending more in the universities, helping

to shape the pattern of research in the image that suits them best. Because the foundation's spending bears more directly on the quality of technical education, in secondary schools as well as in universities, the promised increase cannot fail to be a sound investment. The fact that they may materialize is a feather in the cap of Dr George ("Jay") Keyworth, director of the Office of Science and Technology Policy at the White House. So too is the decision not to leave to Congress the choice of a number to represent the budget of the National Institutes of Health, for there will now be less scope for the dreaded Waxmans to monkey with the constitution of the institutes.

Disappointments

For the rest, where research support is concerned, the new budget is the predicted mixture of small disappointments. The National Aeronautics and Space Administration will continue to gather information about the Solar System more quickly than it can afford to have the data analysed and digested. There will, indeed, be \$150 million to be spent next year on the early design of a space station whose purpose has not yet been decided. There seems to be nothing in the budget to offset the decline of modest overseas collaboration threatened by the proposed withdrawal from the United Nations Educational, Scientific and Cultural Organization (Unesco). Star wars, or preparations for them, are to flourish. The fast breeder reactor has finally been abandoned but for the wrong reason; the Department of Energy should have recognized years ago that the Clinch River design is technically obsolete, but should this year have made provision for the "crash programme" of fast reactor development for which Congress will be crying when it gets to hear of the European plan for cooperation in this field.

These complaints are only minor minuses compared with the outstanding weakness of the federal budget for 1985 — the administration's plan to finance its operations during the coming year by the device on which it has relied since it came to office, the trick of paying its bills with borrowed money. The sums involved are vast, in round numbers \$200 million a year or two-thirds of the enlarged defence budget that Congress is being asked to approve. The federal government's borrowing amounts to 6 per cent of the gross national product of the United States, which is another way of saying that the government used the whole of last year's growth to cover the gap between what it spent and what it raised in taxes. The debt that has accumulated after nearly a decade of this spend-thrift way of running a government has risen so that annual interest payments now exceed 2 per cent of the gross national product, and are reaching the point at which no Secretary of the Treasury can hope to escape from them. The borrowed funds come from two sources, domestic savers (including corporations) in the United States who lend to the government funds that would otherwise be invested in productive industry, and investors overseas who find it profitable to put money into US financial securities while interest rates remain high and who have so far also thought it safe. As the months go by, they increasingly ask themselves whether it is wise to have lent so much on the chance that the federal government will one day be in balance again.

The symptoms of this imbalance are plain, at least from the outside. Because interest rates must be kept high to attract borrowers, the dollar is strong compared with other currencies, US



manufacturers are less able to sell their products on world markets and the United States must run a huge deficit on its trade balance — \$40,000 million last year, probably more in 1984. Even though the flexibility of the US economy has in the past year allowed for growth (by no less than 6 per cent) without accelerating inflation, even President Reagan agrees that this state of affairs cannot continue much longer. His offer last week to negotiate with Congress some way of moving towards a balance (by 1989) is unlikely to bear fruit in the run-up to the election. The question that will increasingly keep people awake at night is whether before then the house of cards will collapse, perhaps because overseas investors in the United States take fright, perhaps because still higher interest rates bring the economic recovery to a stop.

On the face of things, these arcane considerations may seem to have no bearing on the provisions in last week's budget for the support of basic science. But they are crucial. In due course, the federal government will have to balance its books more closely by increasing taxes or by spending less. Either way, the flickering economic revival will be impeded. If spending cuts are the chosen method, who can be sure that the generosity of this year's budget towards basic science can be maintained? If higher taxes bear the brunt, who can be sure that the funds now channelled from industry towards basic science will continue to flow. And if the administration, this or the next, chooses to fudge the issue by allowing inflation to run riot again, who will be safe? □

Time to move Greenwich

On the centenary of the choice of the zero of longitude, other places for it deserve a hearing.

In the week in which Britain, once an imperial power on whose vast possessions, it was said, the Sun never set, has suffered the humiliation of being thoroughly beaten by upstart New Zealand at the national sport of cricket (like baseball, played with pieces of wood and bruising balls, but unlike baseball in that games can last for five days), it is natural that there should have been nostalgic celebration of the centenary of the choice of the meridian through Greenwich as the commonly agreed zero of longitude. Here at least, wishful argument seems to go, is proof that there was a time when the centrality of the British place in the world was generally acknowledged. But even that may be a slightly hollow comfort.

The truth is that Greenwich is far from being the most convenient place through which to trace the zero of longitude. In Western Europe, one of the most densely populated regions of the world, several countries (Britain, France, Spain and so on) find themselves straddling the meridian, unable to decide whether they belong to the Eastern or the Western Hemisphere. Plainly a better choice would have been to place the zero of longitude 10 or even 20 degrees west of where it is at present; the first choice would have left only New Zealand uncertain where it is, the second would have bisected the surface of the Earth in such a way that no country of importance was thus embarrassed. Astronomers, after all, are usually reasonably clever people, and would have been able to assimilate the notion that the zero of longitude should be in the mid-Atlantic just as astronomers in Potsdam and Pulkova have had to learn that they have been well to the east of it.

It is also important, at this centenary, modestly to acknowledge that the conference in Washington in 1884 that settled on Greenwich (with one dissenting vote) let slip a splendid opportunity to put on a more reasonable footing the measurement of angular displacement. The sad truth is that, from sheer indolence and inertia, generation after generation of people who acknowledge the good sense of measuring most quantities on a decimal scale have indolently allowed themselves to continue measuring angles in an arithmetic whose radix, for practical purposes, is 60. By doing so, they have lazily adopted a system variously attributed to the Babylonians and to the ancient cultures of India. It is true, of course, that changing to a system in which, say, radians would be the equivalent of 100 degrees would have been a considerable undertaking, and that it would have been necessary also to decimalize time. But that is no excuse for having done nothing. □

How not to test drugs

British and French anxiety about human volunteers in drug trials should be settled quickly.

THE British pharmaceutical industry, long used to the cultivation of an air of injured innocence in the face of complaints from central government about the prices of drugs sold to the public health services and from sections of the general public that its products are unsafe, has every reason to be aggrieved that some of its arrangements for testing a new drug in volunteers should have called in question the whole basis on which the toxicity of new compounds is assessed (see page 495). The only immediate comfort is that a similar issue has, by coincidence, arisen in France.

The circumstances in which this row has arisen are not nearly as complicated as they seem. In Britain as elsewhere, clinical trials to assess the efficacy and side effects in patients of a potential drug require formal approval from the regulatory body, the Department of Health acting on the advice of the Committee on the Safety of Medicines. But manufacturers, required by established procedures to provide evidence of the toxicity of a new drug by means of tests with animals, also have an incentive to provide evidence that the new material causes no obvious harm to people. The tradition that a person responsible for developing a new material will try it out on himself and his close colleagues (never satisfactory) has in the past few years become outdated as the need has grown for more and more systematic tests in human beings as part of the process of licensing a drug for clinical trials. The result is the practice of delegating testing in volunteers to commercial companies.

Except to those who hold or seem to hold that new medicines as such are an abomination, there is nothing wrong with these procedures, at least when they are followed sensibly. What has gone wrong with the study of a putative tumour inhibitor is that the good sense of the proposal has not been assessed by people at once knowledgeable enough to make an informed judgement, independent in the sense that no benefit accrues to them and publicly identifiable. A commercial company's internal ethical committee whose identity is not disclosed will not suffice when volunteers are recruited publicly, and invites complaints that participants have been less than fully informed of the risks they run. The failure of the company concerned to inform the volunteers' own physicians of what was afoot is another needless omission. But it is sheer humbug that many who should know better have been complaining that by offering participants a cash reward of £250, those mounting the study have been offering inducements great enough to sway a person's judgement of his own long-term interests. It could just as well be argued that a participant without the wit to demand a fee of this order is unlikely to be fit to give informed consent.

The danger now is that the Department of Health will panic at what appears a little like the discovery of a loophole in the arrangements for the regulation of drug testing, introducing another layer of paperwork with which to belabour the drug industry and, worse still, to impede the development of new medicines. The need, however, is far simpler — a publicly agreed set of guidelines by which all drug testers can be bound. The rules should be easily devised. The socially disadvantaged, especially prisoners and old people, should not be allowed to volunteer for drug tests unless, as the French ministry of health suggests, there is reason to think their health may benefit. People who are not sufficiently alert to give informed consent should similarly be excluded. Consent for all drug studies should be given by properly constituted ethics committees, whose names and credentials are known and whose opinions will be made publicly available. Nobody should complain if people or even university departments occasionally make modest sums of money out of these procedures. Now and again, ministers of health should summon up their courage and make public speeches pointing out the need for such activities, now too easily and too often counted as dispensable. □

US budget

Military and basic science offered more rapid growth

Washington

PRESIDENT Reagan released his proposal for the 1985 federal budget last week, and left no doubts about the direction of his research and development policy: massive increases on the military side. The President proposed a record \$33,852 million for military research and development, an increase of 22 per cent over current spending. And while total civilian research and development spending would be held almost constant under the President's plan, its basic research components would increase by 10 per cent overall, with particularly generous treatment for the National Science Foundation (NSF), the National Aeronautics and Space Administration (NASA) and the Department of Energy.

The new money for basic research is to come largely from deep cuts in applied research and — that particular bugaboo of the Reagan Administration's free-market philosophy — the engineering demonstration project. Energy projects, notably solar and fossil, but also (against the administration's will) the Clinch River fast breeder reactor, are the chief victims.

Basic research

Dr George Keyworth, the President's science adviser, has been taking pains to cast this budget as staunchly pro-science. For the first time, basic research is to receive a larger share of total non-military research appropriations than either applied research or development. Keyworth points with pride, too, to new funds for university instrumentation and training and to the fact that basic research support to universities has grown steadily during Reagan's term, reaching nearly \$4,000 million in this year's proposal, an increase of more than 25 per cent in real terms from the level where it had stagnated under the previous administration.

Last week also provided some apparently good news about industry's contribution to US research and development. According to a forecast released by the Battelle Memorial Institute, industrial support is expected to grow by 10 per cent in 1984 to a point where it makes up slightly over half of all US research and development expenditure, public and private.

The emphasis on basic academic research in the President's budget may well stifle complaints that the real increases have been reserved for military projects. If the Reagan budget is adopted, military research and development will have more than doubled (in nominal dollars) in just four years. Most of the military spending supports the new weapons systems to

which the Reagan Administration has committed itself, including the Trident II and MX missiles and the B-1 bomber. A significant share of the new funds, however — roughly \$250 million — is earmarked for the ballistic missile defence programme (also known as Strategic Defense Initiative or star wars).

Science foundation

On the civilian side, NSF is once again in line to be the big winner, with a 14.6 per cent increase in its budget to come on top of last year's 17 per cent increase. Mathematical and physical sciences and engineering would receive the lion's share of the new money, which includes \$10 million for the establishment of "Centers for Cross-Disciplinary Research in Engineering" to focus university research on problems faced by industry and to train graduate and undergraduate engineering students.

The NSF budget also includes a 22 per cent increase in support for scientific instrumentation — a total of \$237 million, \$122 million of which will be available under research grants to individual researchers with the remainder going to support university-based and national centres. Support for graduate students under research grants will increase by 15 per cent, allowing an additional 1,050 students to participate. And a new half-million-dollar experimental programme will provide women scientists with an opportunity to initiate research projects.

NSF has received the go-ahead to begin construction of the Very Long Baseline Array (VLBA), a network of radio telescopes across the United States. VLBA is expected to offer 100 times greater resolution than any existing radiotelescope.

A modest effort in supercomputing — far short of what advocates had hoped for — is also included in NSF's proposed budget. No new hardware will be purchased, but \$20 million is to be made available to pay for access to existing supercomputers and to cover local user costs, such as terminals, software, networks and communication time. In a separate budget item, NSF plans to acquire a new supercomputer for the National Center for Atmospheric Research at Boulder, Colorado.

Energy

The Reagan philosophy is most evident in the proposed budget for the Department of Energy. While overall spending on research and development is to grow by only 1 per cent, basic research is to receive an 18 per cent increase.

Funds will be provided to start construction of the electron accelerator designed by the Southeastern Universities Research Association, which beat Argonne National Laboratory last year in a bitter political fight. Brookhaven National Laboratory, which last year lost another bitter fight to keep its illfated ISABELLE accelerator alive, is to receive its expected consolation prize of \$15 million for a tunnel that will link its existing van de Graaf accelerators and synchrotron, creating a new heavy-ion facility. A plan to incorporate the ISABELLE ring to create a massive relativistic heavy-ion machine is apparently still several years away.

The energy budget also provides support for initial studies of the Superconducting Super Collider concept in order to establish its feasibility and then to produce a specific design in several years.

Keyworth's pet project at Lawrence Berkeley Laboratory, the Center for Advanced Materials, has survived a year of criticism and, having lost its synchrotron advanced light source and the prefix "National", is to receive \$11 million with which to continue construction.

This year, the administration has taken a ►

President Reagan's 1985 budget for research and development, fiscal years (1 October–30 September) 1984 and 1985

	1984		1985
	Reagan request	As passed	Reagan request
NSF	1,240	1,239	1,408
NIH	3,842	4,240	4,342
NASA	2,473	3,257	3,341
Defence-related	29,882	27,636	33,852
Energy	4,713	4,844	4,885
Agriculture	849	872	898
EPA	208	250	281
All others	2,589	2,941	2,769
Research and development facilities	1,195	1,439	1,306
Total	46,991	46,718	53,082

Source: Office of Management and Budget. All figures in millions of dollars.

different approach to its perennial battle with Congress over the National Institutes of Health (NIH).

In past years, the tactic has been to ask for no increase for NIH in the knowledge that Congress in its rapture with that most popular of scientific agencies will add too much anyway. The administration has become increasingly concerned, however, that Congress's enthusiasm tends to be channelled into advocacy for narrow, disease-specific research, a tendency that threatens NIH's traditional freedom to support valuable but less glamorous basic research. "The 'disease-of-the-month club' syndrome is no longer a joke", Keyworth said recently.

This year, the administration is proposing to give NIH an additional \$102 million, but specifically for areas of basic research that it believes have been neglected, such as neurobiology. Keyworth said he hopes that this proposal will "open a dialogue" with Congress and will give the administration a chance to air its views on the importance of basic research in the life sciences — particularly its role in underpinning US competitiveness in

biotechnology.

The Environmental Protection Agency, which in past years has felt the full weight of the administration's budget-cutting, is in for a modest increase this year in research funding. The news may not cheer the environmental lobby, however, as much of the new money will go for further studies of acid rain at a time when many — including a panel that Keyworth himself assembled — have concluded that enough studies have already been done to support regulatory action, a step at which Reagan still balks.

The National Oceanic and Atmospheric Administration (NOAA), the National Bureau of Standards (NBS) and the US Geological Survey are all in for major cuts in support for research and development on the principle that much of their research belongs in the private sector.

Particularly hard hit is NOAA, which is to lose a full one-third of its \$242 million research and development budget. And NBS's fire and building research centres are once again under threat of closure.

Stephen Budiansky

Space science

Panel's advice ignored

Washington

SPACE scientists are unlikely to give more than two cheers for the 1985 budget request for the National Aeronautics and Space Administration (NASA). The agency's basic research receives a hefty increase to \$828 million and three important new projects — the Mars Geoscience/Climatology Orbiter, the Upper Atmosphere Research Satellite and the Scatterometer experiment — have been given the go-ahead. But NASA has unexpectedly balked at a plan to inject urgently needed new funds into university research groups.

The plan, devised jointly last year by university space scientists and NASA officials, carried the *imprimatur* of Frank McDonald, NASA's chief scientist. Yet neither of its chief recommendations found



its way into the 1985 budget. One was to spend \$11 million extra a year for five years to buy new equipment for universities — chiefly oscilloscopes, spectral analysers, computers, micro-ion probes and gas analysers. The other was to resurrect the NASA graduate fellowships that helped build up university space science in the 1960s.

Worse still, the budget contains no increase for basic research grants for university space science. Support for physics and astronomy is held at the same level as last year, while the amount for planetary science is reduced. Only one important recommendation of the McDonald report — more money to analyse mission data — has been incorporated in the 1985 request. Funds for space shuttle operations are due to rise from \$81 million to \$105 million, but that is not expected to be enough to make shuttle science significantly cheaper or faster; since 1978 there has been a backlog of experiments waiting to fly.

NASA's overall budget increase is a scant \$274 million, or 4 per cent. That is enough, however, to keep alive major projects such as the Space Telescope (now renamed the Hubble Space Telescope), the Galileo mission to Jupiter and the Venus Radar Mapper. For 1985 only \$150 million is allocated for research on the proposed space station.

Peter David

Agricultural research

Towards grant competition

Washington

THE US agricultural system, which has been repeatedly criticized for fostering mediocrity and neglecting basic science, is in for a major shake-up if the Reagan Administration has its way. The 1985 budget proposal would triple the size of the small programme of competitive grants for agricultural research, bringing it up to \$50 million, the maximum level currently authorized by law. The bulk of the new money would be reserved for basic research related to biotechnology.

Under the administration's proposal, most of the federal funds for sponsored research in agriculture would continue to be distributed to the country's land-grant colleges according to a state-by-state formula not involving peer review. But by rapidly expanding the competitive grants programme beyond its token level of \$17 million, the administration hopes to effect a major change in the way agricultural research is carried out. The expanded programme is expected to attract researchers at non-land-grant universities (among them the leading US basic research centres, such as Harvard and Stanford Universities) which are shut out from the formula-fund system. It may also bring some scientific rigour, by way of peer review, to a system often accused of drifting more and more to applied research of dubious scientific merit.

The new money, £33 million, is to come in part from an overall increase in the research budget of the Department of Agriculture (USDA) and in part from the phasing-out of the so-called "special

grants", funds earmarked, usually by Congress, for specific projects.

Congress has balked at past efforts to expand the competitive grants programme, which the smaller land-grant colleges in particular see as a threat to their assured support from the formula funds. This time things may be different. Many of the larger and scientifically more competitive land-grant institutions have broken ranks and have declared themselves in favour of an enlarged competitive grants programme. And both Secretary of Agriculture John Block and presidential science adviser George Keyworth have taken a personal interest in the programme.

Besides the new effort in biotechnology (which would receive \$28.5 million), the administration's proposal would establish a small effort in animal sciences (\$4.5 million); existing components in plant sciences (\$15 million) and human nutrition (\$2 million) would continue. Approximately one-quarter of the competitive research funds would be expected to support graduate research assistants working under project grants.

The National Science Foundation's plant biology programme, which has traditionally taken up the slack in basic plant research, is also due for an increase under the President's budget — a 16 per cent rise, to \$58 million.

The USDA formula funding would grow by 2 per cent next year; and Department of Agriculture's own in-house research arm, the Agricultural Research Service, would receive a 3 per cent increase. Beltsville is not to be frozen out.

Stephen Budiansky

US military research

Caltech to expel Army think-tank*Pasadena, California*

A US Army think-tank set up at the California Institute of Technology (Caltech) is to be closed at the urging of the faculty. Growing concern over the think-tank, known as the Arroyo Center for Army Analysis, led last week to an emotional two-hour faculty meeting at which Caltech's president, Marvin Goldberger, was criticized for having allowed the centre to be established under what could be viewed as Caltech's auspices. The faculty urged Goldberger to sever ties with the centre "expeditiously and in a responsible fashion at the earliest possible time".

Goldberger said he would honour the faculty's wishes, but that the university would meet its existing commitments to the Army. The centre could thus keep its ties with the university for up to three years.

The centre is the Army's first outside think-tank. Its mandate is to examine future needs of the Army and to carry out objective analyses of policy questions. It was hoped that association with a leading university would give the centre an air of independence and attract good people.

The Army got its foot in Caltech's door by way of an expanding relationship between the Department of Defense and the Jet Propulsion Laboratory (JPL), the Pasadena space research centre operated by Caltech for the National Aeronautics and Space Administration (NASA). Three years ago, NASA slashed its support for JPL, most of whose planetary exploration missions were shelved. The Caltech faculty, saying it wished to preserve JPL's "unique" research teams, with some trepidation authorized JPL to accept up to 30 per cent of its support from the Department of Defense, and the proportion has indeed grown to 12 per cent (roughly \$50 million of its \$425 million budget).

Ironically, NASA's fortunes have rebounded in the past year, and JPL is now busy with a new mission to Venus and several smaller projects. Nonetheless, Goldberger and JPL's director, Lew Allen (a former Air Force chief of staff), have continued to encourage the military to invest in JPL's future. The Arroyo centre was seen as a "goodwill gesture" to the Army — and a direct link with the Army's upper echelon. The centre now has 23 employees, a director and a separate facility at JPL.

The Caltech faculty began to be concerned about the centre last autumn. "We were told everything was exploratory", said one faculty member, "but real commitments were being made." Last week's "Faculty Discussion" — a special session called only when serious matters need airing — concluded that the Army centre does not fit in with Caltech's strengths and expertise. A particular concern was that Caltech could be seen as lending its reputation to the

policy recommendations made by the centre. Although 60 per cent of the Caltech faculty have individually consulted for the military, 95 per cent say it is wrong for the university to enter into an institutional ar-

US foundations**Kettering changes course***Boston, Massachusetts*

A SMALL drama of scientific tradition confronted by unavoidable change is being played out in Ohio. As part of a major shift of focus of its research, the Charles F. Kettering Foundation of Dayton, Ohio, is arranging to hand over the Kettering Research Laboratory of Yellow Springs to the Battelle Memorial Institute, the large non-profit contract research organization in Columbus, Ohio.

The Kettering Laboratory has built its reputation over the past 50 years on basic research in the plant sciences, concentrating on nitrogen fixation and photosynthesis. It has some twenty senior scientists in a staff of eighty. In the past, 60 per cent of its financial support has been provided by the foundation, most recently to the tune of about \$1.6 million a year, while the remainder has been made up of federal grants and some contracts.

The foundation announced the new arrangement in May 1983, and Battelle took over management of the laboratories last September. Assuming that the Internal Revenue Service approves the merger, the deal will be completed in the next few weeks. The Kettering Foundation will provide \$8 million over the next five years to maintain current operations during the laboratory's adjustment to becoming part of Battelle.

Battelle, a public trust whose charter states that it must "benefit mankind", and whose net income is disbursed to charity through the Battelle Foundation, carries out applied contract research for clients in such areas as metallurgy, information sciences and organic chemistry. Battelle is otherwise similar to a profit-making business: it must generate income by selling its work to government and industry.

The expected changes are worrying the laboratory's staff, used to functioning independently in an academic atmosphere and focused primarily on long-term non-applied questions. One leading investigator asserts that he and about half of the senior staff at the laboratory are seriously hunting for other jobs. Under the new regime, members of staff will deal with novel concepts such as "deliverables" and "marketing", anathema to many of them, and will be responsible to Battelle management.

Albert Adelman, associate director of Battelle Columbus, claims that he and his staff are trying to make the changes as easy

as possible for those at the laboratory. Battelle has asked everyone at the laboratory to stay on. It is consulting the staff about major decisions, particularly in the search for a new scientific director, and new people with more experience in business will be phased in gradually.

Goldberger said that there had been a breakdown in communications with the faculty on the issue and that "all the worries should have come up sooner". He said that Caltech and the Army have yet to work out the details of how the centre will be operated in the light of the faculty's recommendations. **Sandra Blakeslee**

as possible for those at the laboratory. Battelle has asked everyone at the laboratory to stay on. It is consulting the staff about major decisions, particularly in the search for a new scientific director, and new people with more experience in business will be phased in gradually.

The question remains of why the Kettering Foundation decided to divest itself of the Kettering Research Laboratory. Kettering is an "operating foundation", meaning that more than 80 per cent of its endowment income is channelled into its own projects. In addition to the laboratory, the foundation has supported social science research, including an international affairs programme, the Institute for Development of Educational Activities and an urban management programme. All three have either been gutted or spun off since 1981, when David Matthews, Secretary of Health, Education and Welfare under President Gerald Ford, became president of the board of trustees.

According to Robert Daley, director of public affairs at the foundation, the decision to transfer the laboratory to Battelle was made by the trustees to "ensure the long-term growth of the laboratory". He claimed that Battelle was chosen because it would be better for the laboratory to be associated with a larger international company with wide experience in research management. He says that the laboratory staff were informed of the decision-making process, but at least two senior researchers insist that the announcement of the merger plan last May was a complete surprise.

Meanwhile, the Kettering Foundation has turned its attention to matters of public policy. It now supports a Domestic Policy Association whose goal is to foster public understanding of federal policies. It is also developing other programmes to educate the public about government, says Daley.

The Kettering Laboratory was founded in the late 1920s by the successful inventor and entrepreneur Charles F. Kettering "to sponsor and carry out scientific research for the benefit of humanity", particularly in plant science and agriculture. Despite the foundation's contention that it is maintaining Kettering's original trust, many staff members feel betrayed by what they see as abandonment by the foundation and its trustees of its essential purpose.

Christopher Earl

European information technology

Only one dog left in manger

Brussels

WEST Germany will agree to contribute its share towards Esprit, the £1,000 million Brussels-inspired European research programme on information technology, at a meeting of research ministers later this month. So, at least, European Commission sources believe after the visit to Bonn by research commissioner Etienne Davignon two weeks ago.

In the past six months, Britain and West Germany together having been blocking progress on the project pending a wider European agreement on the budgetary problems of the European Communities. But now, it seems, Britain may be the only obstacle.

According to Brussels, West German opposition was limited to tying a release of Esprit funds to an offsetting reduction of the whole Brussels research budget (which is dominated by energy, including nuclear fusion). But, it seems, Bonn has been convinced that such a move would be counterproductive: the Brussels research directorate has recently been putting its house in order, and, Davignon argues, is ready to take on more work.

Thus, with West Germany ostensibly out of the way, Davignon is turning all his attention to Britain where the nut will certainly be harder to crack. Not only has the United Kingdom set up its own information technology programme under the Alvey directorate (on a scale roughly equivalent to what Britain might get out of Esprit), but the British Prime Minister insists on a complete solution to the European budget issue before new spending is approved. Davignon seeks to tackle this policy at its source.

Ironically, the Esprit sums for 1984 are already written into the current Brussels budget, but cannot be released until the ministers agree. Frustrated, the Commission is already unofficially preparing the official "call for tenders" that would follow ministerial approval. The hope is that after 28 February (when research ministers will meet in Brussels) the Esprit motor will be warmed up and running, just waiting to leap off the grid in its race against Japan and the United States.

Meanwhile, the European Commission has managed to get at least one new technology project under way: an eight-laboratory effort, costing £1 million, to produce the semblance of a working, optical computer within two years.

Esprit, although considered advanced and pre-competitive by industry, does not include "optronics", considered by some as the real future of computing and by others as a mere dream. But Esprit has now been all but taken over by the Brussels industry directorate, leaving the research directorate to look further ahead.

This it certainly has done. One goal of optronics is a thousandfold increase in computing speeds (to picosecond rather than nanosecond switching times) but there is at present no demonstrable read-write memory or clock working at these rates. But the optronics programme, which has been under way for little more than a month, will put Europe ahead of the United States and Japan in these fields, according to one of its co-directors, theorist Dr P. Mandel of the University of Brussels.

The eight groups involved, at Heriot-Watt University (Glasgow) (where Dr S.D. Smith is director of the experimental part of the European project), Milan, Pisa, Strasbourg, Frankfurt, Freiburg, Munich and Brussels, knew of one another before the Commission programme was mooted, but they are now working together with a will, claims Mandel. **Robert Walgate**

Soviet computing

Aleksandrov urges speed

ATTEMPTS to computerize the Soviet economy could turn out to involve "vast and futile expenditure", Academician Anatolii P. Aleksandrov, president of the Soviet Academy of Sciences, has been warning. Writing in *Izvestiya*, Aleksandrov alleges that the Soviet Union is failing to make proper use of "even the comparatively small amount of computer equipment" that it manufactures.

The main bottleneck, according to Aleksandrov, is the shortage of trained personnel in the Soviet Union and a lack of awareness of the potential of computers among the population at large; overcoming these deficiencies will be a task comparable with "eliminating illiteracy" after the revolution.

Aleksandrov's article is ostensibly a call for a massive training programme, with the necessary computer manuals produced "before 1985", tuition in computer sciences (already partially implemented in tertiary education) at the secondary level and salary incentives for employees who complete computer familiarization courses.

Computers and automation equipment, Aleksandrov urges, must be "highly reliable", with at least 3-5 years of "trouble-free working". Accidental power failures, he said, should not lead to loss of information or to damage.

The situation with software is more complicated. The introduction of computers into the Soviet economy has taken place piecemeal, so that the various designers and ministries have produced equipment which, although "quite good for its time", is incompatible in software and components.

UK data protection

Reservations over data bill

THE British Government's Data Protection Bill successfully ran the gauntlet of its second reading debate in the House of Commons last week, despite Labour opposition and public reservations by several professional bodies.

The bill, which has been controversial from the start (see *Nature* 302, 641; 1983), sets out to prevent the abuse of personal information held in machine-readable form so as to allow Britain to ratify the Council of Europe's convention on data protection. But many fear that the vague terms employed in the bill and the wide scope of its exemptions from this laudable aim will make it a burden on companies which would be required to register as "data users" while providing no worthwhile protection to people about whom data are held, "data subjects" as they are called.

The need therefore is for a major overhaul of the Soviet computer industry so that all future hardware and software would be compatible. The logistical implications of such a revision of plans would be considerable — particularly in the Soviet context, where quarterly, annual and quinquennial production targets make no allowances for retooling or changes of production lines. If a switch to fully compatible hardware and software is decided, the planners will be hard pressed to incorporate it into the directives for the 1986-1990 five year plan.

The technical quality of existing Soviet software, to judge from Aleksandrov's remarks, is not entirely satisfactory. Existing tasks in the software sphere, he says, include the adaptation of software to automatic search systems and the protection of software against distortion due to voltage or frequency fluctuations, electromagnetic interference and the like.

For Aleksandrov, however, the task is clearly that of education. His programme for computer education (if the comparison with the literacy campaign of the 1920s has any real meaning) would embrace about half the population of the Soviet Union. Such a programme would itself require a major coordinated effort and, according to Aleksandrov, even the production of computer education manuals ("before 1985") must be preceded by work by the State Committee for Science and Technology, the Academy of Sciences, all ministries manufacturing computer equipment and automation facilities and the ministries of higher and secondary education, in order to "integrate" the available technical resources and software. **Vera Rich**

The several amendments agreed by the House of Lords have not been enough to satisfy the critics. Holders of personal data stored in machine-readable form — subject to certain exemptions — would be required to register with a new Data Protection Registrar, who would keep records on the sort of information being held. (Personal data, as defined in the bill, include expressions of opinion about individuals but not indications of data users' intentions regarding those individuals.) Data subjects — subject to further exemptions — would be allowed access to information held about them and would be entitled to demand that any inaccuracies be corrected. Data users, for their part, would in general be prevented from unauthorized disclosure of personal information — again, subject to exemptions.

It is the exceptions to the general principles of the bill that arouse anger. The main concern of the British Medical Association is that computerized medical records would, if the bill became law, have to be registered, presumably by the health authorities, whose representatives — including non-medically qualified personnel — would then be empowered to pass on such records either to the police or to the Inland Revenue. The medical profession considers this quite unacceptable.

The British Medical Association (BMA) and a medical inter-professional working group on personal information have been negotiating with the Department of Health to find a solution to the problem. The department has offered some concessions based on voluntary codes that would in any case apply only while records were within its purview. BMA and the working party want medical records to be specifically exempted from the general provisions of the bill so that they could only ever be disclosed to an outside authority on the order of a crown court judge. At the same time the bill must not interfere with epidemiological research.

An earlier blanket exemption from the bill's subject access provisions and prohibitions on disclosure for data relating to immigration control has now been dropped, but it seems that the Home Secretary retains the power to exempt much government-held data from subject access "if they appear to him to be of such a nature that their confidentiality ought to be preserved". Universities are worried about the possibility of having to hand over their students' academic records on demand. And although data subjects who do manage to establish that inaccurate information about them is being held do not now have to prove damage in order to be awarded compensation, this does not apply if the data are improperly disclosed, a surprising omission picked up by Liberal Member of Parliament Mr Simon Hughes.

Quite apart from these sensitive issues, many fear that the proposed system for control of computerized personal data would be quite unworkable. The Data Pro-

tection Registrar would probably have a staff of about 20; the number of data users who would have to register would be of the order of hundreds of thousands, many of them holding entirely innocuous information. The definitions in the bill are so vague that it is unclear whether many computer systems will be required to be registered or not. The bill contains no detailed guidelines or codes of practice to help data users, and some Members of Parliament have suggested simple devices that would enable the letter of the bill to be complied with while breaking its spirit — for example, maintaining different (but unlabelled) lists for (say) creditworthy and uncreditworthy customers.

Those who defend the bill point out that

as there is now no protection against the abuse of personal data in British law, the bill must at least be an improvement. But this would be to forget that once an important bill becomes law, major modifications are unlikely to be made for some years afterwards. Liberal and Social Democrat Members of Parliament who voted for the bill say they will be pressing for further changes in the committee stage. But the committee will be very limited in the expert opinion on which it will be able to call, and a Labour move to put the bill before a special standing committee was heavily defeated. However, the Home Secretary appears willing to accept that further modifications to the bill will be necessary.

Tim Beardsley

Fast reactors

Anglo-French accord at last

THE British and French electricity utilities have agreed on an outline programme of fast reactor development.

Sir Walter Marshall for the Central Electricity Generating Board (CEGB) and M. Jean Guilhamon for Electricité de France (EDF) signed a document on Tuesday which "sets out principles for long-term cooperation" covering the joint construction of fast reactors. According to CEGB, the first such joint reactor would be built in France, while the locations of future stations have yet to be decided.

In the short term, this implies CEGB support for the building of Superphénix II, a putative successor to the 1,300 MW Superphénix I now nearing completion near Avignon. Previously, EDF has shown itself cool to the Superphénix II project because of its probable cost (about twice that of an equivalent pressurized water reactor (PWR)).

Sir Walter Marshall said on Monday that the CEGB contribution to a Superphénix II would be "neither large nor trivial", and certainly more than 16 per cent. CEGB would receive electricity and revenues in proportion.

Assuming this more advanced reactor would cost no more than 40 per cent more than an equivalent PWR, the electricity so bought by CEGB would be cheaper than electricity made by burning coal in the United Kingdom, said Sir Walter.

According to M. Guilhamon, EDF is aiming to start Superphénix II in 1986. "Breeder reactors are easier to operate than PWRs" said Guilhamon. Breeder operators suffer less exposure to radiation, and the thermal inertia of the sodium cooling circuit offers greater protection against accident, Guilhamon claimed. The sodium is unpressurized, and normally runs at 500°C; but it must reach 1,000°C before it boils. The water in a PWR, however, is superheated. This gives a PWR a 20 minutes safety margin but a fast breeder reactor two hours, he claimed.

Sir Walter would be happy to see just one

British demonstration fast reactor before 1997 (his retirement date), he said. The main constraint was not cost but the "exhausting" prospect of a public inquiry probably longer and larger than the current Sizewell inquiry into the proposed British PWR. This has been "psychologically very difficult" said Sir Walter. Sizewell must be well out of the way before another such inquiry were contemplated.

Meanwhile, further agreements between British and continental agencies are expected, following the outline inter-state agreement on fast reactor cooperation signed in January. One between the UK Atomic Energy Authority (UKAEA) and its European partners is taking longer than promised — but as this shares out the research among signatories, and as research is the main component of fast reactor work at present, the delay is not surprising. A UKAEA spokesman pointed out wryly that while the CEGB-EDF agreement was between only two agencies, the research agreement involves several in a number of countries.

In fact, some years ago UKAEA had been seeking just such a bilateral agreement — but with the United States rather than France. The authority hoped that the trouble over federal support for the Clinch River project would lead the United States to fall into the arms of a British collaboration; but the US fast breeder programme was finally seen to be so confused and irretrievable that the decision was made to join in with Europe.

It may be considered significant that Sir Walter Marshall, who as the then chairman of UKAEA was behind the approach to the United States, has now also concluded a bipartisan agreement (though this time with France) as chairman of CEGB. Sir Walter approves of clarity, and it may have been against his taste to negotiate with a plethora of European agencies (as are involved in the existing European consortium behind Superphénix I).

Robert Walgate

UK drug trials

Row over student trial

EVENTS have forced the British Medicines Commission into an urgent study of arrangements for testing new drugs on healthy volunteers. The issue has come to a head in the wake of complaints that plans for testing a new antitumour drug among London students are ethically objectionable and pay scant attention to the safety of the volunteers.

The disputed study, begun some months ago but now abandoned, was being carried out by a company known as Charterhouse Clinical Research Unit Ltd, on behalf of the manufacturer, Ortho-Cilag Pharmaceuticals Ltd. Volunteers, mostly students,

Rabin, professor of biochemistry at University College, London, who says HPR may plausibly accelerate the growth of some tumours, and who complains that Charterhouse had provided no information to the volunteers' own physicians and that it had no plans to follow up its volunteers to detect long-term untoward effects. He also attacks the information sheet given to volunteers for failing to list all possible side-effects of HPR and says the silence of Charterhouse's consent form on the matter of compensation in the event of damage being suffered is "totally disgraceful".

These criticisms are rejected by Professor Paul Turner, an adviser to Charterhouse and director of clinical pharmacology at St Bartholomew's Hospital Medical College. He says that "ill-considered remarks" and "destructive press coverage" have severely impeded research on a promising anti-tumour drug "showing no signs of toxicity".

Turner says that Charterhouse accepts work only on the condition that a written indemnification against damage claims is provided by the client company. A new consent form being drawn up by Charterhouse will aim to comply with a recommendation of the Association of British Pharmaceutical Industry that no-fault compensation should be offered explicitly to volunteers. Turner argues that new drugs such as HPR are best tested in an independent company separate from the manufacturer.

Although the HPR study had been ap-

proved by Charterhouse's own ethics committee, the deans of the London medical schools have now recommended that their students should take part only in studies that have been subject to independent scrutiny. Under a new agreement between Charterhouse and the Metropolitan Conference of Medical Deans, studies on students will in future be approved by the ethics committee of St Bartholomew's Hospital, with which Charterhouse is closely associated.

Although many British pharmaceutical manufacturers have in-house testing units, more and more volunteer studies have recently been carried out by small independent companies, often associated with pharmacology departments in medical schools, whose earnings can provide a welcome source of research funds. Charterhouse Clinical Research Unit Ltd was set up by Synthelabo, a non-profit charity linked to St Bartholomew's Hospital Medical College.

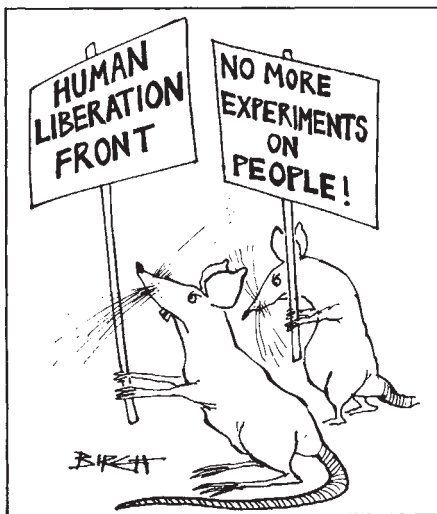
The demand for volunteer studies has grown rapidly since the introduction in 1981 of the Clinical Trials Exemption Scheme, which allows manufacturers with a good case for a drug to go into clinical trials without first facing a full-length scrutiny by the Department of Health's Committee on the Safety of Medicines. It is widely believed that a good volunteer study increases the chances of an exemption certificate being granted.

Practice varies from one company to another: some, such as Drug Development (Scotland) Ltd, linked to the University of Dundee, routinely notify volunteers' physicians about studies. Dr John McEwen, the company's medical director, says that information from volunteers' physicians has often proved useful to those running the studies. The company also offers no-fault compensation in the event of claims for damages, and is insured to cover any claim.

The Medicines Commission, which advises the government on matters related to medicines generally, now has some difficult questions to face. Members will certainly be mindful of Professor Turner's warning that if drug development is hampered in this country, the big manufacturers will simply pack their bags and go elsewhere.

Manufacturers feel hard done by because more is expected from them and from their pharmacological agents than from academic researchers in, say, physiology. Thus in the event of harm to a volunteer in an experiment by an employee of the Medical Research Council, the council would offer an *ex gratia* payment but there would be no legal redress unless negligence could be proved. The voluntary code on compensation to which most commercial companies adhere commits them to damages in a way the Medical Research Council and the Department of Health have been unwilling to apply to themselves.

Tim Beardsley



were paid £250 to be given 20 consecutive doses of up to 450 mg of hydroxyphenylretinamide (HPR), an analogue of vitamin A shown to be a tumour inhibitor in animals.

The study, designed to provide information of toxicity in human beings, has been criticized on the grounds that volunteers were not fully informed of the risks and that the testing company had failed to make adequate arrangements for monitoring participants' health. Inevitably, the result has been to reopen the contentious question of how clinical data on the effects of new drugs should be gathered — and of the role of academic pharmacologists in the process.

Under present arrangements in Britain, drug studies with healthy human volunteers are regarded as a private matter between consenting adults — the drug company and the volunteers. Since 1981, manufacturers have not been required to seek advance approval from the Committee on the Safety of Medicines (established, like the Medicines Commission, under the 1968 Medicines Act). One result has been the emergence of a number of companies linked with academic departments to provide human toxicity testing as a service to pharmaceutical manufacturers.

The whistle seems to have been blown on the Charterhouse trial by Professor Brian

France seeks rules

THE testing of new drugs in France is to be tightened up, in a move by the ministry of health. No precise bill is yet on the table, but a "draft project" is under study, according to the French evening newspaper *Le Monde*.

French law on the matter is obscure, with some experts claiming that human tests are totally illegal. Testing has nevertheless been carried out, but often in a clandestine and unsatisfactory manner, making France — according to some — an effectively liberal regime in which multinationals can conveniently try out new formulas.

According to the ministry's draft, however, subjects and the authors of trials should sign clear written agreements with each other; payment should be according to published terms; authors would have the burden of proof; and prisoners, or members of the author's staff, would not be used as subjects unless it would be to the benefit of their health. Further, all human trials would be subject to the approval of a regional ethical committee — though the constitution of such a body remains, for the moment, undefined. Robert Walgate

Sahel drought

Call for joint action

A CALL for the formation of a Sahel fund to combat the area's drought and desertification was issued last week at the end of the sixth meeting of the Permanent Interstate Committee for Drought Control in the Sahel (CILSS), at Niamey, the capital of Niger. The committee was formed ten years ago by eight West African governments (Cape Verde Islands, Chad, Gambia, Mali, Mauritania, Niger, Senegal and Upper Volta) to coordinate action during severe droughts. The sixth meeting was attended by heads of governments and the director-general of the Food and Agricultural Organization.

The World Meteorological Organization considers that the drought in Africa which began in 1968 has not yet ended, and is worst in the Sahel. Agencies working in the area report that although conditions in the Sahel are not everywhere as serious as in 1968-73, drought is now locally severe in the north. For example, in Mauritania, 85 per cent of the basic cereal crop has failed, with the result that half the population is now gathered in the capital of Nouakchott. In northern Mali, all cereals are badly affected. In Upper Volta, millet has failed entirely and the sorghum crop is poor. So a return to the conditions of the 1970s is feared. Ghana, with its third consecutive poor harvest and an increase of 10 per cent in population since the expulsion of Ghanaians from Nigeria, is expecting a fourfold increase in food prices within six months. In Senegal the cereal situation is disastrous, with animals dying and water holes drying up. But in the southern areas, harvests have been adequate and there are even some surpluses.

Water is the main problem. It is estimated that only 25-30 per cent of Sahelians have access to sufficient water. Desertification, which according to the United Nations Environment Programme, consumes 1.5 million hectares of potentially productive land a year, is exacerbated by population growth (2.7 per cent a year) and overgrazing.

Wood consumption (90 per cent of it for burning) is also a worry, especially because attempts at reforestation have not been encouraging. The area could show a substantial surplus if consumption were reduced by even one per cent.

The effects of physical problems are made worse by the chronic social and economic conditions of the region, including inadequate transport, low farm prices, which discourage the production of food, and a general insufficiency of money. Emergency aid is difficult to deliver.

According to the Club du Sahel, formed by the Organization for Economic Cooperation and Development and several West African governments and various



Countries affected by the drought in the Sahel region include Mauritania, Senegal, Mali, Upper Volta, Niger, Nigeria, Chad and Ghana.

development agencies, aid to the area has doubled in ten years and now amounts to 15 per cent of the total gross national product in the Sahel states and more than 20 per cent of the gross national product in half of them. So dependent on aid has the region become that some agencies now fear that long-term massive food aid is sapping the ability of the region to acquire the resources necessary to respond to normal climatic swings, let alone to cope with disaster. Oxfam, the British charity, agrees with the UK Overseas Development Administration that the best help is the swift delivery of emergency food aid followed by agricultural aid, such as the supply of seed corn, as conditions improve.

One novel possibility is that climatic change and crop yield may be predictable from satellite data (see p.528 for a study of satellite results); this would be especially valuable if there is a recurring pattern comparable with the 10-year cycle now established for Botswana. A British project is currently under consideration by the

Overseas Development Administration and the Food and Agricultural Organization. The World Meteorological Organization, however, considers that drought cannot be predicted and that there is no practical method of enhancing precipitation, but that much could be done to ameliorate the effects of droughts by improving agrometeorological monitoring services and the wide application of increased information on an interdisciplinary basis.

Conditions in the Sahel are part of a climatic threat to the world's poorest people. Nine Southern African states also made an emergency appeal last week. Oxfam, in common with other international agencies, has received more requests for help in the past six months than at any time in its entire 40-year history. Abnormal weather, Oxfam says, could lead to "the collapse of development across a whole sweep of countries so economically depressed that they cannot possibly cope themselves".

Sarah Toozee

Nature index of biotechnology stocks

12-Month high	12-Month low	Company	Close previous month	Close 27 Jan.	Change
23 1/4	9 3/4	Biogen (Switzerland)	10 3/4	12 1/2	+ 1 3/4
6 1/4	1 5/8	Bio-Logicals (Canada)	1 5/8	2	+ 3/8
16 1/8	7 1/4	Bio-Response (USA)	10	11 3/4	+ 1 3/4
19	10 1/2	Cetus (USA)	10 7/8	13 1/8	+ 2 1/4
15 1/2	6 1/2	Collaborative Research (USA)	6 3/4	8 1/4	+ 1 1/2
39 7/8	15	Damon (USA)	15 3/8	17 3/4	+ 2 3/8
34 1/4	16 3/8	Enzo-Biochem (USA)	25	23	-2
18 7/8	8 3/8	Flow General (USA)	9 1/2	8 3/4	-3/4
49 3/4	25 7/8	Genentech (USA)	34 1/2	37 1/4	+ 2 3/4
17 3/4	7 7/8	Genetic Systems (USA)	8 7/8	8 3/8	-1/2
23 1/4	12 1/2	Genex (USA)	13	13 1/2	+ 1/2
31	18 3/4	Hybritech (USA)	18 3/4	21	+ 2 1/4
22 1/4	10 7/8	Molecular Genetics (USA)	12	14 1/2	+ 2 1/2
23 1/4	10 1/2	Monoclonal Antibodies (USA)	12	12 1/2	+ 1/2
73 1/2	42	Novo Industri A/S (Denmark)	56	55 3/8	- 5/8
30 1/4	13 3/8	Pharmacia (Sweden)	19 3/8	18 7/8	-1/2

Closing prices are for the last Friday of the month. For over-the-counter stocks, bid price is quoted; for stocks on the American and New York exchanges, the transaction price. *Nature's* weighted index of biotechnology stocks stood at 181 on 27 January, compared with 175 a month earlier. Data from E.F. Hutton, Inc.

The day before

SIR — In a leading article in *Nature* of 1 December 1983 (p.412) it is recommended that ABC show the film "The Day After" again, and then again, in order that people do not forget the effects of an all-out attack with megaton weapons which have been extrapolated from the known effects of the 20 kiloton explosions over Hiroshima and Nagasaki.

I was struck by what seem to me to be significant omissions about what happened the day before: the actors portrayed in the film made almost no organized effort to improve their chances of survival. There are two scenes showing men shovelling small quantities of earth against the foundations of a church and a farmhouse, while there are innumerable scenes showing people panicking in city streets, grocery stores and on the highways. In one scene Dr Oats, the senior surgeon-haematologist, washes his hands at a scrub sink while an orderly tells him, in a tone of voice appropriate for the revelation that skinned cats have been found in the hospital kitchen, and that there is a rumour that people are leaving Kansas City. The question is asked, "Where does one go from Kansas City?"

It would have been even-handed and more interesting had the producers of the ABC film shown that the people of Kansas City could have moved into the large complex of commercially developed caverns which underlie the city and its environs. These caverns were artificially excavated out of limestone to provide crushed rock for the Kansas and Missouri interstate highways. The excavated areas are covered by between 80 to 200 feet of bedrock and soil, have an even temperature (57–62° F), a large area (several hundred acres) and are connected to each other and to the surface by many miles of roads and railroads. The Monsanto and Libby food companies have stored very large quantities of canned food and frozen fruit juice in the caverns, and other companies have stored cars, tractors and vodka. It has been estimated that everyone in Kansas City is within 45 minutes of one of the entrances to these caverns, and that the caverns could be used for blast and fallout protection¹.

Few cities have the opportunities for evacuation comparable with those of Kansas City, but then few cities are located beside 150 Minuteman missile silos. It has been estimated that an all-out attack with megaton weapons would kill or injure 50–80 per cent of the unprotected population of the United States, and that the destruction of hospitals would make it impossible for physicians to "intervene effectively in the (resultant) human injury and death"^{2,3}. These speculations have been repeated so often that many authors accept them as fact^{3,4}. However, these estimates assume that urban populations remain unprepared and unprotected. Most of the

casualties from megaton weapons would result from the blast effects, and by removing people from the likely target areas, evacuation could substantially reduce the number of deaths⁵.

In a broader context, it is critically important that the superpowers and their allies keep talking in order to reduce as much as possible the number of nuclear weapons and the likelihood that any of those weapons will ever be used. Until such talks have borne generous fruit, the nations of the Northern Hemisphere would be wise to emulate Sweden, Switzerland and the Soviet Union and improve their civil defence capabilities.

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Crucifixion date

SIR — I read with great interest the review article by Humphreys and Waddington entitled "Dating the Crucifixion" (*Nature* 306, 743–746; 1983). The date of 3 April, of the year AD 33, the 14 Nisan of the Jewish Calendar, appears to be well supported by their evidence. But one of their statements may be in error. They write "... we note ... that the Crucifixion must have occurred on 14 Nisan and that the interpretation of the Last Supper as a Passover meal held at the official time cannot be correct". However, it is likely that the official time for the Passover meal in AD 33 was on the night of 13 Nisan. In support of this view I present the following paragraph from p. 326 of *Jesus of Nazareth* by Joseph Klausner (Macmillan, New York, 1944).

"According to the ruling which was newly promulgated by the Pharisees in Hillel's time, the Passover was regarded as a public sacrifice; if, therefore, the 15th of Nisan fell on a Sabbath and the 14th on the eve of Sabbath, the Passover was sacrificed on the eve of Sabbath (the 14th of Nisan) at the moment "between the two days", even if this profaned the Sabbath; they used to argue that, like every public sacrifice, "the Passover abrogates the Sabbath rules." According, however, to an earlier ruling, which held good among the priestly party almost to the close of the period of the Second Temple, the Passover was regarded as a private sacrifice and one which might not abrogate the Sabbath rules; if, therefore, the 14th of Nisan fell on the eve of Sabbath, they sacrificed on the 13th instead of the 14th, so as not to profane the Sabbath (since they must sacrifice "in the evening at the moment of sunset.") Hence Jesus and his disciples celebrated Passover on the Thursday, the 13th of Nisan, and during the ensuing night of the 14th of Nisan (the night before Friday) they had to celebrate the "Seder", the Passover meal, with its unleavened bread and bitter herbs, instead of on the night of the 15th Nisan."

BORIS MAGASANIK

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Animal rights

SIR — Since you have established from recent correspondence that animal rightists are not only cranks but humourless ones to boot, it is without relish that I enter the fray. With one conclusion of your leading article (*Nature* 306, 527; 1983) I can entirely agree: there are no consistent or universal principles that imbue animals with "rights". A right, then, is not a given, an absolute, but is a function of the culture which accords it. Any right is a more or less widely accepted value built up over time and representing something of which we consider an animal or human should not be deprived.

While I can see no evidence for absolute natural rights of animals, to retain these for our own species while withholding them from other animal species is, in my view, antediluvian — or, to be more accurate, pre-Darwinian. What distinguishes this viewpoint from the ethnocentricity of those tribes whose members believe that they are the only humans and that people of neighbouring tribes can be killed like pigs?

Sir Andrew Huxley, president of the Royal Society, is reported to have asked what importance can be attached to the painless killing of purpose-bred laboratory animals that otherwise would not have lived. Man, according to this belief, has a right to destroy because he fancies that, like a god, he can create life. The surgeon Versalius used to say "I dressed him; God healed him." His antidote — a little humility.

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City slickers

SIR — The proposal for an Indian "technology city" (*Nature* 305, 350; 1983) is welcome, but it is unfair to build such a city exclusively for Indian scientists working abroad. It is important to recruit the best of Indian scientific talent, no matter whether those concerned work in India or in the United States. While some of the best Indian scientists are working in India, not all Indian scientists working abroad are of high calibre.

I think the Government of India should go ahead with its plans but that it should appoint the best persons in the field, whether they are working in India or abroad. This could be achieved by a selection board comprising top ranking experts from India and other nations. There is always a risk that the very best scientists are not offered jobs in India for various sociopolitical reasons, but something has to be done if India is to keep pace with international developments.

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Cell and cancer biology meld

The startling similarity of part of epidermal growth factor and the product of a viral oncogene is another leap forward in understanding the basis of cancer. A definite link to human cancer remains elusive.

FOR some time, there has been speculation that many viral oncogenes would turn out to be derived from cellular genes for growth factors or their receptors, so when Waterfield *et al.*¹ and Doolittle *et al.*² showed, seven months ago, that one of the two peptides that constitute platelet-derived growth factor (PDGF) is similar in sequence to the transforming protein encoded by the *sis* oncogene of simian sarcoma virus, there was eager anticipation of the next example. That has now materialized with the paper on page 521, in which Waterfield and his associates³ reveal that an oncogene of a tumour virus of birds is almost certainly derived from the gene for the cell surface receptor for epidermal growth factor (EGF). The result is as dramatic as it will be important for research on normal and abnormal (cancer) growth of cells. But it is not as simple as it appears at first sight.

The EGF receptor gene has been a good candidate for a viral oncogene because⁴ it shares with at least five viral oncogenes (of which the prototype is *src* of Rous sarcoma virus) the activity of a tyrosine phosphokinase, that is an enzyme which phosphorylates tyrosine residues in proteins. It has therefore been on the cards that one of the tyrosine phosphokinase encoding oncogenes would closely resemble the EGF receptor gene. If so, the argument went, the tumour virus would in effect be placing an EGF receptor in an inappropriate cell and/or at an inappropriate time. In the event, neither idea is exactly borne out.

The sequence of the *erb-B* oncogene of AEV-H, a strain of the avian erythroblastosis virus that causes erythro-leukaemia and, less frequently, sarcoma in chickens, was published last November by Yamamoto *et al.*⁵. Unexpectedly, it contains a segment that is notably similar to the *src* oncogene and others with tyrosine phosphokinase activity. Nevertheless, at least three separate laboratories have since failed to demonstrate that the *erb-B* oncogene product has tyrosine phosphokinase activity of its own (an anomaly shared with the *mos* oncogene⁶ and, very recently, the *fms* oncogene⁷). But while the tyrosine phosphokinase activity of EGF receptor has been known for some years, the sequence of the receptor has awaited its purification in sufficient quantities.

Starting either with human placenta or with the human epidermoid carcinoma cell line A431, and with the vital assistance of

monoclonal antibodies against the EGF receptor, Waterfield's group has now determined the amino acid sequence of 14 fragments of the receptor. These scraps of information have been sufficient for a computerized data search to align six of the peptides with segments of the protein encoded by *erb-B*, with 74 identical amino acids out of 83. The other eight peptides could not be aligned with *erb-B*.

Waterfield *et al.* conclude that the *erb-B* oncogene represents only part of the EGF receptor gene — cautiously, they raise the formal possibility that it comes not from the receptor gene itself but from a closely related gene — and deduce that the missing part encodes the EGF-binding domain of the receptor which is positioned on the external surface of cells. What the gene retains is the short segment of EGF receptor that spans the membrane and the large segment on the internal surface of the membrane which has tyrosine kinase activity.

The first puzzle is why, then, does *erb-B* not have demonstrable tyrosine phosphokinase activity? One possibility is that it does, but that the activity has been missed, for lack of rigorous testing. If so, the next puzzle is how it can be stimulated when the *erb-B* version of the EGF receptor lacks the EGF-binding domain and the normal receptor has low tyrosine phosphokinase activity in the absence of EGF. A clue to the solution to both puzzles may come from the recent demonstration by Tamura *et al.*⁸ of the activation of the tyrosine phosphokinase of insulin receptor by the proteolytic action of trypsin; perhaps the truncation of EGF receptor really does activate it.

It is even conceivable that the tyrosine phosphokinase activity, or lack of it, is not wholly relevant to the issue. Could it be that other activities of viral oncogenes/growth factor receptors are more relevant to cancer? For example, is the recently demonstrated induction of the *myc* gene of lymphocytes as an early response to PDGF⁹ a more relevant clue or does that induction anyway depend on the tyrosine phosphokinase activity that PDGF induces in its receptor?

The next question that is likely to be answered by Waterfield and his associates is the intellectually more modest but technically formidable one of what the complete sequence of the EGF receptor looks like. The answer will be derived from the nucleotide sequence of its messenger RNA converted into cDNA. The sequencing is already well under way at Genentech under

Axel Ullrich, an author of Waterfield *et al.* The sequence will reveal more clearly the exact relationship between the EGF receptor and the viral *erb-B* gene product. Moreover, a comparison between the cDNAs of EGF receptor and those of the cellular *erb-B* gene¹⁰ will make it easier to decide whether the two genes are identical.

The relevance of the discovery of Waterfield *et al.* for cancers, particularly human cancer, not caused by tumour viruses simply cannot be answered at this stage. It is, at least, clear that activation of the cellular *erb-B* gene by avian leukosis virus, a tumour virus of chickens that does not carry its own oncogene, can cause a variety of neoplasms, particularly erythroblastosis¹¹. Therefore, by analogy with *myc* genes^{12,13}, it is possible that the human cellular *erb-B* gene can be inappropriately activated by, for example, chromosome translocations. A thorough comparison of the expression of *erb-B* EGF receptor genes in healthy and tumour tissues should at least indicate how frequently they are expressed in abnormal amounts or in abnormal sizes (perhaps as a truncated protein) in tumours; the A431 cancer cell line was a good starting point for the EGF receptor purification precisely because it has at least fifty times more receptor than normal cells.

The discovery will also act as a spur to the purification and sequencing of other growth factor receptors, especially those with tyrosine phosphokinase activity — the PDGF, insulin and insulin-like growth factor-1 receptors. Perhaps it is from their genes that some other viral oncogenes have been derived. But it will be necessary to look further afield to account for them all, particularly if the recent identification¹⁴ of about one-third of the gene for actin as part of the oncogene of a tumour virus of cats is relevant to its ability to produce fibrosarcomas.

Peter Newmark

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Neurobiology

More nerve growth factors?

from Joshua R. Sanes

THE discovery of nerve growth factor (NGF) by Levi-Montalcini and Hamburger¹ was the first major step in the molecular analysis of neural development, and remains the most influential achievement in this rapidly growing field. This soluble protein is required for the survival of sympathetic and sensory neurones *in vivo* and *in vitro*: in culture, it also promotes neuronal differentiation and chemotactically directs growing axons. NGF has been purified and sequenced, its genes are being cloned and mapped, and studies of its mechanism of action are underway. The current dogma is that NGF is produced by peripheral target tissues, picked up by nearby axons, and transported back to neuronal somata. Competition for limited supplies of NGF may determine which neurones live and which die during embryogenesis. In addition, NGF may act locally in the periphery as a stimulant and attractant, to regulate the size and shape of an axon's terminal field. Finally, NGF may be required for neuronal maintenance in adulthood, with loss of its supply — after axon injury, for example — signalling the neurone that adjustment or repair is required (reviewed in refs 2–4).

The early realization that only a few types of neurone are sensitive to NGF led to speculation that NGF was but one of a family of trophic agents⁵. If each of several cell types turned out to secrete limited amounts of a factor that supported its own innervation, retrograde trophic control would provide a general means to match the sizes of pre- and post-synaptic populations. Furthermore, a system of selective trophic sustenance might play some limited part in synaptic specificity: that is, in determining which cells or connections, as well as how many cells or connections, form or survive. Among the first steps in the hunt for other trophic factors have been the demonstration that extracts of appropriate targets support parasympathetic neurones *in vitro*^{6,7}, and the purification of a protein from brain that allows survival of some cultured neurones that do not respond to NGF⁸.

From a cell biological point of view, the most useful new factor to obtain would be one that supports somatic motor neurones — a motor neurone growth factor (MNGF). The skeletal neuromuscular junction is the best studied of all synapses and would be a good system for studying factor uptake and action. Fortunately, there is reason to suspect that a MNGF exists, in that several aspects of neuromuscular synapse formation seem to reflect control by muscle-derived factors. In the embryo, spinal motoneurones (like many other neuronal types) are normally

produced in excess; 30–50 per cent of them degenerate before the animal is born. Peripheral control of this process is indicated by the observation that the naturally occurring cell death can be increased by extirpation of the appropriate target, while neurones that would otherwise die can be rescued by grafting on extra target tissue. In the neonate, several axons innervate each skeletal muscle fibre. Excess inputs are withdrawn until each fibre receives a single input, and it has been proposed that even after a neurone's survival has been assured, its individual axonal branches continue to fight for the limited support that each muscle fibre can furnish. In the adult, partial denervation of a muscle induces the undamaged axons to sprout and reinnervate the denervated muscle fibres. Again, several aspects of the sprouting phenomenon suggest that it represents an axonal response to soluble factors released by denervated fibres. Thus, neuromuscular connectivity could be regulated in part by the supply and/or effectiveness of MNGF(s) throughout the life of an animal (reviewed in refs 9–12).

How, in turn, might the supply of such growth factors be regulated? Again, previous studies of muscle suggest a candidate: electrical (or contractile) activity. Paralysis of muscle rescues embryonic motoneurones destined to die, slows elimination of polynervous innervation in neonates and evokes sprouting in adults; conversely, chronic stimulation speeds synapse elimination and prevents sprouting^{8–11}. Since many aspects of muscle metabolism are already known to be activity-dependent, it is tempting to suppose that denervated or inactive muscles secrete more MNGF than innervated, active muscles. Neurones could thus control and be controlled by their supply of factor, establishing a feedback loop.

Aware of these and other issues, many groups have begun to search for a MNGF. The most common approach has been to culture motor (or spinal cord) neurones in a minimal medium, and to assess the effects of adding muscle extracts or muscle-secreted material. Supplements have been found that increase levels of neurone survival, neurite extension, or transmitter synthetic capacity^{13–21}. As one might have hoped, extracts are more active when made from neonatal or denervated muscle than from innervated adult muscle^{18–21}. Early attempts at fractionation suggest that the extracts may contain several active factors, which differ in their modes of action (some seem to act in solution, others after first attaching to the substratum) and effects (for example, activities that promote neuronal survival and differentiation can

be separated from each other). However, purification of the factor(s) has not yet been achieved.

So far, all attempts to identify a MNGF have relied on neurones in culture as bioassay. Now Gurney²² reports, in this issue of *Nature* (p.546), results of an alternative immunological approach. He used a series of antisera (originally prepared at the MRC, London) to material secreted by organ-cultured denervated muscle. To carry out an assay he injected the sera into paralysed but innervated mouse muscles and looked for suppression of the sprouting that would normally occur. Gurney's paper contains three intriguing results. First, several antisera partially prevented sprouting *in vivo*. Second, each blocking serum recognized a 56,000-molecular-weight (56K) protein on immunoblots of muscle-secreted material. Third, and perhaps most astonishing, sera from patients with amyotrophic lateral sclerosis (ALS) not only blocked sprouting when injected but also recognized a 56K antigen on immunoblots. ALS is a disease in which many spinal motoneurones die and surviving motoneurones sprout poorly. Thus, the properties of ALS sera both provide a possible clue to the aetiology of this fatal disease and lend further support to the notion that a 56K protein may regulate sprouting.

Is the 56K antigen a MNGF? A promising preliminary result is that the antigen is present in an incompletely purified fraction that promotes neurite extension and survival of cultured spinal cord cells (ref. 22 and personal communication). If further work proceeds well, the idea that neurones of different types and of different ages are maintained in a trophic equilibrium will be open to new and exacting tests. □

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Solar conversion

Non-aqueous electrolytes make wet cells work

from S. Dennison

IN the urgent search for photovoltaic devices for the direct conversion of solar energy to electricity, solid-state solar cells have in the past few years far outstripped their counterparts based on junctions between semiconductors and electrolytes. Although 'wet' photovoltaic cells may be more easily manufactured, they are far behind as practical converters. But now there is a prospect that this gap may be closed.

At the end of 1982, Stanford University chemists Chris Gronet and Nathan Lewis described (*Nature* **300**, 733; 1982) a cell based on an n-GaAs_{1-x}P_x semiconductor in contact with a non-aqueous redox electrolyte with an efficiency of no less than 13 per cent, a doubling of the best previous efficiency. The problems that had plagued earlier devices were overcome by optimizing the semiconductor properties and preventing corrosion by the use of a non-aqueous electrolyte. Now, Gronet and Lewis, together with C. Lieber, have made an equally impressive step forwards by the construction of a 10 per cent efficient cell based on the rather more familiar material p-type silicon, in a non-aqueous electrolyte containing the cobaltocene-cobaltocenium redox couple (see this issue of *Nature*, p.533).

The efficiency of 10 per cent, obtained both with a laboratory lamp and direct sunlight, dwarfs that of previous cells based on p-silicon, putting it high in any league table of 'wet' photovoltaic cells. At the same time, the Stanford group has demonstrated a major departure from the expected behaviour of p-type silicon in contact with a non-aqueous electrolyte and have shown how certain intrinsic limitations on cell performance expected from the behaviour of the semiconductor-electrolyte interface are not apparent in their cell.

To a large extent, the major problems of the liquid-junction photovoltaic cell (see our discussion, *Nature* **300**, 687; 1982) have been solved by the replacement of aqueous with non-aqueous electrolytes. By the rigorous exclusion of water, semiconductor corrosion is almost completely suppressed. These solvents also allow a far wider range of redox couples to be used, so that difficulties of poor electron-transfer kinetics can be overcome. But the use of non-aqueous media introduced a further complication — an apparent limitation of the photovoltage obtainable from a cell, a phenomenon now known as 'Fermi level pinning'.

The phenomenon is this. With a number of semiconducting materials, the observed photovoltage seems to be more or less

constant for each material and independent of the redox couples in solution with which it may be in contact. But on the conventional model of such an interface, the photovoltage should reflect the difference between the redox potential in solution and the Fermi level of the semiconductor. The observation that the photovoltage is substantially constant thus implies a constant difference between the redox potential and the Fermi level, which in turn suggests that the redox couples in the non-aqueous medium are in some way 'pinning' the Fermi level.

The explanation lies in the charge distribution at the interface. On the conventional view, there should be a depletion layer beneath the surface of the semiconductor across which most of the potential difference between the bulk semiconductor and the bulk electrolyte should be dropped. A change in the redox potential should then alter the potential gradient within the depletion layer and thus the photovoltage. But if there are electronic states at the semiconductor surface which can be charged and discharged, the potential gradient at the surface will be substantially different from that expected and the interface more like the junction between a metal and an electrolyte. Such surface states may arise from bulk defects in the crystal lattice, from dangling bonds at the surface or by the adsorption of charged species onto the

surface. The overall result is a characteristic photovoltage for each junction between a semiconductor and an electrolyte which is independent of the redox couple. It has recently been thought that behaviour of this type is common among small band-gap semiconductors, precisely those which are of most use for solar conversion, p-type silicon in particular. So Fermi pinning and the consequent loss of photoconversion efficiency has been regarded as the last nail in the coffin for liquid-junction photovoltaic devices.

The latest results from the Stanford group are particularly important because they show that device performance is not necessarily limited by the intrinsic properties of the interface between p-Si and the non-aqueous electrolyte. The explanation is not yet clear, but it seems likely that there is an obverse phenomenon to 'Fermi pinning' which might be called 'redox-pinning'. In a photovoltaic device of the type described, both oxidized and reduced forms of the couple would accumulate at the surface. If the components of the redox couple can communicate with the bulk of the semiconductor much faster than the surface states, the filling of these states will be effectively determined by the relative concentrations of the components of the redox couple in solution. And if these vary little with the degree of bias on the semiconductor, then the junction will behave as a classical device.

It now remains to be seen whether these important findings can be translated into a working cell configuration without loss of conversion efficiency. □

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Molecular biology

Selfish DNAs with self-restraint

from W.F. Doolittle, T.B.L. Kirkwood and M.A.H. Dempster

TRANSPOSABLE elements are small discrete genetic elements which can be replicated independently of the chromosomes in which they are embedded. Mechanisms of independent replication may vary, but the results are almost always the same — new copies appear at new genomic positions without loss of original copies¹. Models of how transposition could occur have been with us for some time and now we are beginning to see models of how the process could be regulated.

In a recent issue of *Cell*, Kleckner and her colleagues describe the inwardly and outwardly directed promoters of IS10-Right, one of the two copies of the insertion sequence IS10 which form the terminal repeats of the bacterial transposon Tn10 (refs 2,3). Transcription of the IS10-encoded transposase gene initiates at the

weak inward promoter pIN. The stronger pOUT is responsible for activation of genes distal to the site of IS10 or Tn10 insertion. The two are transcriptionally independent, but active pOUTs inhibit, in *trans*, the expression of transposase genes. This translational inhibition is mediated, Simons and Kleckner³ suggest, "by direct pairing between the transposase messenger RNA and a small, complementary, regulatory RNA specified by the IS10-encoded pOUT promoter".

This molecular model makes excellent sense of the genetic phenomenon of multicopy inhibition — the suppression of the transposition-related functions of a single Tn10 transposon by multiple copies of its IS10 terminal repeats elsewhere in the cell. It also makes evolutionary sense. Transposable elements are viewed by some as

molecular parasites^{4,5}. Good parasites might wish to be gentle with hosts. (This is, for instance, the textbook explanation for the rapid loss of effectiveness of the myxomatosis virus introduced to control Australian rabbit populations in the 1950s — an attenuated parasite spreads more slowly but more surely through a larger and healthier host population⁶.)

The eukaryotic transposable element P, an inhabitant of the *Drosophila* genome, provides another intriguing example of self-restraint at the molecular level⁷. Some 30–50 partial or complete copies are borne by *Drosophila* strains designated P. The mating of P males with females from element-free M strains produces sickly and nearly infertile ('dysgenic') hybrids as a direct result of unrestrained P replication. Hybrids which do survive produce, over several generations, flies of increasing vigour with increasing tolerance to mating with P males. The hybrid stock has itself become P, and is said to have acquired P cytotype.

There are many ways this could be explained but the recent model of O'Hare and Rubin⁸ has particular appeal. P encodes, they suppose, a transposase and a regulator of transposition. The latter not only affects transposase activity (or synthesis) negatively, but its own activity (or synthesis) positively. Thus "in a dysgenic cross, P factor DNA is introduced into a background where there is neither transposase nor regulator, and expression of both functions leads (at least initially) to transposition. With time, positive feedback by the regulator of its own level of activity produces enough regulator to shut off transposition and the P cytotype is established".

The evolutionary value of self-restraint in transposable elements seems obvious intuitively, but it is always reassuring to have intuition confirmed by theory. The mathematics can be made fairly simple⁹. If χ is the average copy number of the element per cell, the net rate of multiplication for each cell in a (potentially) exponentially growing host population can be written as an expression in three terms, $\rho(\chi) = \phi(\chi) - \gamma \frac{d\chi}{dt} - \omega$. $\phi(\chi)$ is the rate of cell division, which may be diminished as the element load increases; γ is the probability that each new transposition causes lethal mutation; and ω is the basal level of cell mortality independent of the element load. For an element without any form of restraint, both χ and $d\chi/dt$ will increase exponentially with time and in due course $\rho(\chi)$ becomes negative. This may be due partly to declining $\phi(\chi)$, but mainly to rapidly increasing $\gamma d\chi/dt$. Once $\rho(\chi)$ is negative, host cells are dying faster than they are born so the host cell population is driven to extinction and with it the element itself. On the other hand, if the element acquires the trick of restraining its transposition so that its copy number is limited to n , then (provided $\phi(n) > \omega$) the cell multiplication rate remains positive as $d\chi/dt$ goes to zero. The upshot is

that, although the element may impose some small loss of fitness on its host, measurable as $\phi(0) - \phi(n)$, both of them continue to exist.

It takes little insight to deduce that, given these two alternatives, the better strategy for the transposon in the long run is to exercise self-restraint. The selection mechanism by which this outcome is reached is not entirely straightforward, however, since the force at work is the comparatively weak evolutionary process of group selection¹⁰; that is, it is through competition between groups, or clones, of cells containing both types of element that the unrestrained ones are eliminated and the restrained ones arise. Should competition arise within a clone, as would happen if a *Tn10* pIN mutated to become unresponsive to the inhibitory effect of pOUT, the unrestrained mutant would enjoy a temporary but strong selective advantage until the clone

was extinguished. Theory predicts that this type of behaviour should from time to time be observed, and it will be interesting to see if this is the case. □

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Waves and particles: the diffraction of electrons

LOUIS DE BROGLIE sparked one of the most productive controversies in twentieth-century physics when he suggested that a frequency could be associated with a fundamental particle of matter as though it consisted of a group of waves. Could wave-particle duality be experimentally demonstrated?

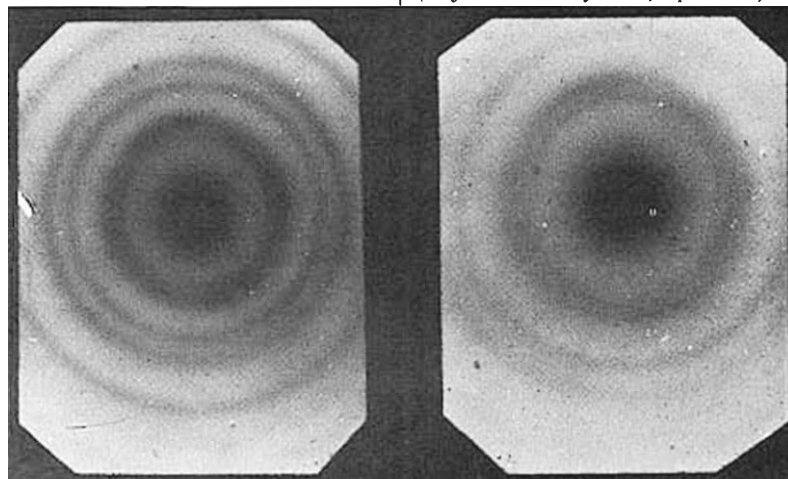
Two independent experiments performed in 1927 implied that it could: the first, a study by Clinton Davisson and Lester Germer, of the scattering of a narrow beam of electrons off a nickel crystal; the second, by George Paget Thomson, of the diffraction of electrons transmitted through thin films. Their respective detectors were a galvanometer and a photographic plate. In Thomson's first experiment, electrons from a cathode-ray tube traversed an extremely thin film of celluloid 10 cm in front of the photographic emulsion, the whole apparatus being evacuated to avoid attenuation of the electron beam by air molecules. A pattern of concentric rings certainly appeared, but the argument for electron diffraction was not

quite watertight since the atomic structure of cellulose was not yet accurately known.

Gold foil was substituted to provide a crystalline lattice of known structure, and shown here are the photographs which Thomson initially obtained (October 1927 at the University of Aberdeen). Larger rings (right) are associated with lower energies, as measured by the spark gap of the induction coil. The patterns, wrote Thomson later, "at once gave rings which agreed quantitatively with the theoretical expectation for a face-centred cubic lattice of side 4.06 Å". He concluded that the detailed agreement between his studies of electron diffraction and the de Broglie theory "must, I think, be regarded as strong evidence in its favour".

The photograph is reproduced by courtesy of the Science Museum (Natural History), where Sir George Thomson's original apparatus is also displayed. □

Selected from Beyond Vision by Jon Darius (Oxford University Press, April 1984).



Gene expression

DNA methylation — how important in gene control?

from Adrian P. Bird

Is DNA methylation a key element in the regulation of vertebrate gene expression, or does it play a minor part? There is support for both views in the recent literature, for while it is now clear that *in vitro* methylation inhibits the transcription of several genes (see *News and Views* 296, 602; 1982)¹, some *in vivo* studies raise doubts that reduced methylation is always a necessary precondition for gene expression.

Experiments with mouse retroviruses have provided strong evidence that DNA methylation can suppress transcription. Integrated viral sequences that are endogenous to mice are non-infectious and heavily methylated. This natural situation can be mimicked by introducing unmethylated proviral DNA into preimplantation embryos or embryonal carcinoma cells². No infection results, and the surviving integrated copies of the provirus are found to be heavily methylated. Purified DNA from each of these sources is non-infectious, but infectivity can be restored by treating the cells with 5-azacytidine, an inhibitor of methylation, or by recovering the integrated provirus after cloning in bacteria³⁻⁴. Since molecular cloning causes loss of methylation at CpG sequences — the major site of methylation in animals — it seems likely that lack of infectivity is caused by methylation.

Confirmation is provided by the observation that remethylating the cloned proviral DNA with a rat liver methyltransferase abolishes infectivity once more⁵. As well as rounding off the experimental logic, these studies make the important point that methylation is inhibitory only at certain CpGs, as methylation of a subset of CpGs with the *HpaII* methylase does not inhibit infection. Since *HpaII* is commonly used to analyse methylation of genomic sequences, it is salutary that, in this case, *HpaII* sites alone were not relevant to expression.

Experiments by Busslinger *et al.*⁶ also stress the importance of specific CpG sequences. Members of the human β -globin gene family were methylated at CpG by using the ingenious method pioneered by Stein and co-workers⁷. The globin gene is cloned in a single-stranded M13 vector and a second strand is synthesized in which all cytosine residues are methylated. Upon transformation of this DNA into mouse cells, cellular methyltransferases can act on the unmethylated strand only at CpG. Thus when the transformed clone grows, the methylation at CpG sequences is maintained while methylation at cytosine residues in other sequences is lost. The initial experiments

showed that cells which have been transfected with both a methylated γ -globin gene (which becomes methylated at all its CpG sequences) and an unmethylated β -globin gene (which remains unmethylated) were able to express the β -globin gene but not the γ gene. Thus methylation appears to be inhibiting transcription.

To extend this result, Busslinger *et al.* synthesized molecules in which certain segments were protected from methylation. When regions upstream of the 5' end were left unmethylated, but the transcribed region was methylated, transcription of the γ -globin gene was seen. But when this pattern of methylation was reversed, so that the 5' flanking regions were methylated, transcription was abolished. These and other experiments establish that somewhere between 760 base pairs (bp) upstream and 100 bp downstream of the 5' end of the γ -globin gene are one or more CpG sequences whose methylation prevents transcription in mouse cells. Methylation at CpG sequences elsewhere is apparently irrelevant to transcription in these cells.

Of course, in order to be sure that loss of methylation is really part of globin gene activation *in vivo*, it is necessary to find out whether the relevant CpG sites are ever methylated in cells which do not synthesize globin. This is particularly important given that the hamster adenosine phosphoribosyl transferase gene is inhibited *in vitro* by methylation⁸, but has recently been discovered to be unmethylated at the relevant sites in most, if not all, tissues⁹. Consistently unmethylated domains, regardless of expression, have also been observed at the 5' end of the mouse dihydrofolate reductase gene⁹ and the chicken $\alpha 2(I)$ collagen gene¹⁰, suggesting that changing levels of methylation are not a component of regulation for these genes either.

The existence of genes with what may be constitutively unmethylated 5' domains does not contradict the idea that DNA methylation interferes with transcription, but merely emphasizes that absence of methylation is not sufficient to ensure transcription. There is, however, a case where heavy methylation of a purified gene appears to have no effect on transcription¹¹. At the time in embryogenesis when the genes neighbouring them become active, ribosomal RNA genes (rDNA) of *Xenopus laevis* lose CpG methylation at certain spacer sequences. In sperm rDNA, though, the CpG sequences of the spacer region, including all 19 sites in the promoter, are highly methylated. Even so, sperm rDNA is transcribed just as effec-

iently as cloned (that is, unmethylated) rDNA upon injection into the nuclei of *Xenopus* oocytes; and transcription does not seem to be due to loss of methylation following injection. By contrast, methylation of two viral genes inhibits their transcription in the oocyte^{12,13}. Although rDNA may not qualify as a typical vertebrate gene, methylation and transcription clearly are not always inimical.

Other exceptional genes have been hinted at by experiments in which methyl-sensitive restriction endonucleases were used to follow hormonally induced changes in gene expression. In the case of the chicken vitellogenin gene, transcription after treatment with oestrogen is associated with loss of methylation at a CCGG site about 600 bp upstream of the 5' end¹⁴⁻¹⁷. Other sites in the vicinity of the gene remain methylated regardless of transcription. What is surprising, though, is that loss of methylation at this *HpaII* site is gradual and follows, rather than precedes, the peak of vitellogenin gene transcription. Moreover, the same *HpaII* site is gradually demethylated in oviduct, a tissue that does not transcribe the vitellogenin gene. In another species, *X. laevis*, all 20 testable CpG sequences at the A2 vitellogenin gene are as highly methylated in hormone-stimulated liver as in unstimulated liver and blood cells¹⁸.

On the face of it these findings seem to violate the conventional view that undermethylation is a necessary precondition for transcription. There is, however, an acknowledged weakness in this kind of experiment — only a minority of CpG sequences are within a sequence that can be recognized by one of the methyl-sensitive restriction endonucleases. Thus failure to find a correlation between expression and methylation may arise simply because the

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endonucleases do not detect the relevant sites.

How can the inherent weaknesses of the restriction enzyme approach be overcome? The answer may lie with an unlikely-sounding, but recently perfected, technique for sequencing unique stretches of DNA in unfractionated genomic DNA. George Church of Harvard University has used a sophisticated blend of blot hybridization and Maxam-Gilbert¹⁹ sequencing to locate all 5-methylcytosine residues in a region of the globin gene of transformed mouse cells (see ref. 4). It would now be interesting to use this technique to monitor other CpG sequences during activation of the vitellogenin genes.

In evaluating recent data on gene expression and DNA methylation, it may be helpful to take an evolutionary perspective, starting from the observation that the level of CpG methylation is considerably higher in vertebrates than in invertebrates. Apart from the much quoted case of *Drosophila*, in which methylated cytosine residues have not been detected at all²⁰, other invertebrates generally have a minor fraction of heavily methylated DNA in their genome²¹. No clear example of a methylated gene has yet been detected in invertebrates. Since these animals nevertheless exhibit cellular determination and differentiation, it seems likely that, in animals, DNA methylation was not involved in the evolution of these basic mechanisms.

Why, then, are the majority of vertebrate genes methylated? One possibility is that DNA methylation in invertebrates has a purely suppressive effect — simply being used to condemn sequences to transcriptional silence — while in vertebrates, the spread of methylation has elicited mechanisms that allow genes to tolerate and, in some cases, to exploit methylation for the control of expression.

There are three ways in which a gene might circumvent suppression by DNA methylation. First, key sequences might be maintained in an unmethylated condition by rendering them refractory to methylation, as in the case of genes whose 5' domains are unmethylated in expressing and non-expressing tissues alike^{8,9}. Second, genes may tolerate methylated bases at their regulatory sequences, as the ribosomal RNA genes of *Xenopus* appear to do. Last, expression of the gene may be suppressed by methylation in inappropriate tissues, but in tissues where the gene is to be expressed transcription can proceed following demethylation. It is this role for methylation that has hitherto attracted most interest, and it is to this category that the globin genes may belong.

A demethylation step is usually envisaged as a pre-requisite for transcription of genes regulated by loss of DNA methylation. A weakness of this view is that to regulate genes separately, a distinct mechanism is required to demethylate each kind of gene — it is

difficult to see why this should have evolved. An alternative control mechanism, and one whose evolution is perhaps easier to envisage as an ancestral gene moved from the unmethylated to the methylated condition, is that activation of the gene begins with the gene still in a methylated state. Activation then leads to demethylation which, in turn, relaxes control of the gene. Here, demethylation is not thought of as a mechanism to facilitate initial activation of the gene, but to simplify continued expression once activation has occurred, perhaps by bringing the gene under more 'everyday' control mechanisms.

Although we do not yet know how any

eukaryotic gene is regulated, preliminary evidence suggests that the degree of involvement of DNA methylation is variable. This is consistent with the view that methylation of genes was a relatively late occurrence in animal evolution, and it suggests that we should not expect a coherent unified mechanism for control of gene expression based on DNA methylation. In order to learn just how much of gene control is due to modulation of DNA methylation patterns, we must await future studies on a gene-by-gene basis. □

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Biotechnology

Time for plant cell culture?

from M. W. Fowler

WITH the announcement last year by Mitsui Petrochemical Industries of a process for the production of the pharmaceutical and dye shikonin by large-scale plant cell culture, plant cell biotechnology at last became a reality on the industrial scene. Although the scale of activity (some 750 litres bioreactor capacity) is small in comparison with large-scale microbial fermentation processes, the shikonin process nonetheless marks an important first step forwards in plant cell biotechnology. Hitherto this area of biotechnology had been considered by many to be 'just another way of looking at plant biochemistry' rather than as a genuine alternative way of manufacturing plant natural products.

Plant cell culture may prove of particular value in the future in situations where a new chemical structure with interesting properties has been identified but the source plant is particularly difficult to grow. Of course, the possibility of deriving the compound by organic synthesis must be considered first — but there again there are now several reports of the synthesis by cell cultures of chemical structures that are quite unique. Before cell culture sets off in new directions, however, the technology must first be proved on an existing product.

In Japan, the Japanese Salt and Tobacco Monopoly has made particular progress towards the development of a tobacco biomass system and has used conventional stirred tank bioreactors (fermenters) at volumes in excess of 6,000 litres. Pharmaceuticals have been the target in West German laboratories and substantial success has been achieved at the University of Tübingen in the development of a process for the biotransformation of low-value digitoxin to the important and high-value cardiotonic digoxin.

Plant cell cultures are maintained in liquid culture in much the same way as yeasts and other microbial cells, but there are some major differences: plant cells

grow much more slowly than microbial cells and doubling times of the order of 20–150 h are typical. Sub-culture periods are thus much longer. Also, it is easy to maintain microbial species in a culture bank, either lyophilized or cryopreserved, but similar techniques are only just being developed for plant cell cultures. In consequence, large liquid culture banks are needed to support plant cell cultures. Even if cell preservation techniques do become viable, the long time required to grow up plant cell cultures still means that careful planning is essential to initiate large-scale cultures.

On present-day costings, plant cell culture is likely to be considered only for high-value low-market-volume products. In general, products have to have a whole sale value of at least \$250–500 per kg to make them worth considering as targets for a cell culture process. Once one or two processes have been developed successfully, however, the increased know-how and confidence are likely to lead to a widening of the target window.

What are likely to be the key developments in the years ahead? The comparatively few large-scale systems that are being actively tested have not encountered major problems. Fairly sophisticated technologies such as fluidized bed and immobilized cell systems are also showing rapid development. One area which is in urgent need of consideration is downstream processing, the unglamorous but often highly expensive end of the business. It is back in the cell however that the biggest problems still face us. Lack of the fundamental biochemical and genetic information that would enable control of product yields is the chief constraint on the development of plant cell biotechnology and there is much fundamental research that needs to be done. □

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Deep seismicity and modes of deformation in Tonga subduction zone

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The geometry of deep (>400 km) earthquakes in the subducting western edge of the Pacific plate, when related to the local distribution of seismic activity, reveals a complex pattern of planes of shear failure, enabling the modes of deformation by which the descending plate shortens and thickens to be identified. The deepest part of the slab is displaced laterally, suggesting that it fails to penetrate the discontinuity at 670 km.

DEEP earthquakes occur in steeply dipping zones—Benioff zones—which, with the advent of plate tectonics, have been recognized as the trajectories of oceanic lithosphere subducted deep into the Earth's mantle¹. Although most Benioff zones terminate at shallower depths, there is an absolute cutoff in activity at 650–700 km, and this has often been taken as *prima facie* evidence that subduction does not penetrate into the lower mantle. It is possible, however, that the cessation of seismic activity corresponds to the point in pressure–temperature space where catastrophic failure ceases to be a possible mechanism of deformation, in which case no inference can be drawn concerning the subduction of material to greater depths^{2,3}. The question of whether or not subduction continues into the lower mantle is of great importance for our understanding of mantle convection and for models of the chemical evolution of the Earth because of its bearing on the question of whether the upper and lower parts of the mantle are isolated, separately convecting systems, or whether they are parts of a single, mantle-wide and chemically mixed convective flow^{4–7}. For this reason we have studied the seismicity in one of the deepest and most active Benioff zones—the Tonga Arc—to obtain information concerning the deformation of material at great depth.

There has been much research related to the possible mechanisms of deep earthquakes. It has long been recognized that under conditions of temperature and pressure appropriate to the mantle, brittle failure is an unlikely mechanism for deep earthquakes and other mechanisms by which shear failure can occur have been put forward^{8–11}. The occurrence of sudden phase transformations in the subducting lithosphere has been considered as a possible mechanism^{12–15}, or as a triggering mechanism¹⁶, for deep events; the seismic evidence, however, favours a mechanism dominated by shear failure, as is the case for shallow earthquakes^{17–19}.

From seismic radiation it is possible to identify a pair of orthogonal planes at the focus—the nodal planes—which are the possible planes of shear failure. Extensive studies have revealed that the orientation of these planes is indicative of systematic shortening or extension in the down-dip direction^{17,20}. However, the existence in deep Benioff zones of persistent planes of failure, or faults, has been an open question. Such planes may be expected to manifest themselves as lineations in the spatial distribution of hypocentres, but evidence of this kind has been obtained for only a few events^{21–24}.

In the Benioff zone associated with the Tonga trench, earthquakes almost invariably indicate down-dip shortening. We have determined^{25–27} the moment tensors of 49 events in Tonga at depths >400 km, using the 'centroid-moment tensor' (CMT) technique of Dziewonski *et al.*²⁸. This technique uses digitally recorded waveforms from the Global Digital Seismograph Network (GDSN) including all body wave phases; this enables us to obtain reliable results for the moment and the

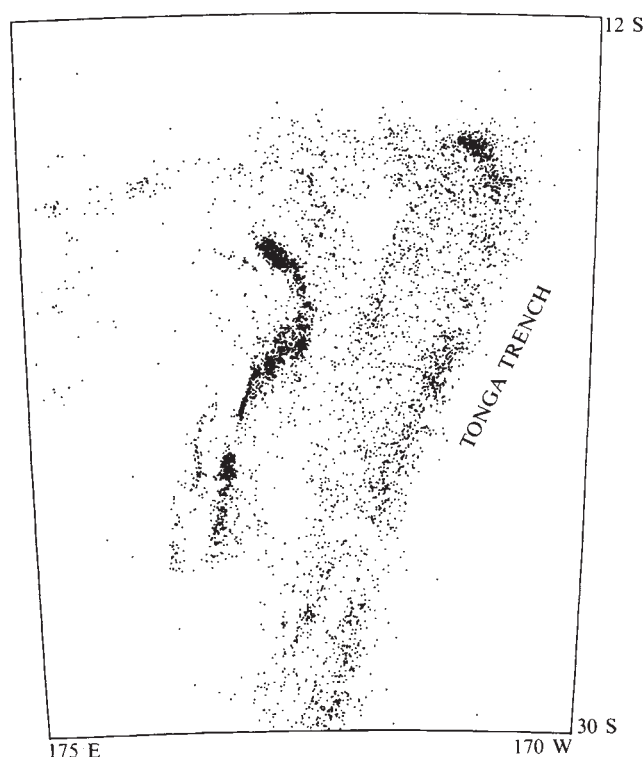


Fig. 1 Map view of the south-west Pacific showing earthquakes with body wave magnitude $m_b > 4$ from the ISC catalogue 1964–79. All the events in the Tonga Benioff zone deeper than 300 km have been relocated using the technique of joint hypocentre determination.

geometry of seismic sources using, in some cases, as few as six stations. An adequate digital data set has been available only since 1977 and the 49 CMT events constitute all earthquakes in Tonga deeper than 400 km large enough for the CMT technique to be applied.

To obtain an accurate representation of the seismic activity in the neighbourhood of each CMT event we have relocated some 2,200 events, deeper than 300 km, using P-arrival times reported by the International Seismological Centre (ISC, 1964–1979). Relocation was performed using the technique of joint hypocentre determination^{29,30} (JHD). Estimates of the reliability of each hypocentre were obtained, and events having poorly determined coordinates were rejected.

One of our principal observational findings is that for more than 40 of the 49 CMT events it is possible to identify planar alignments in the pattern of relocated hypocentres in

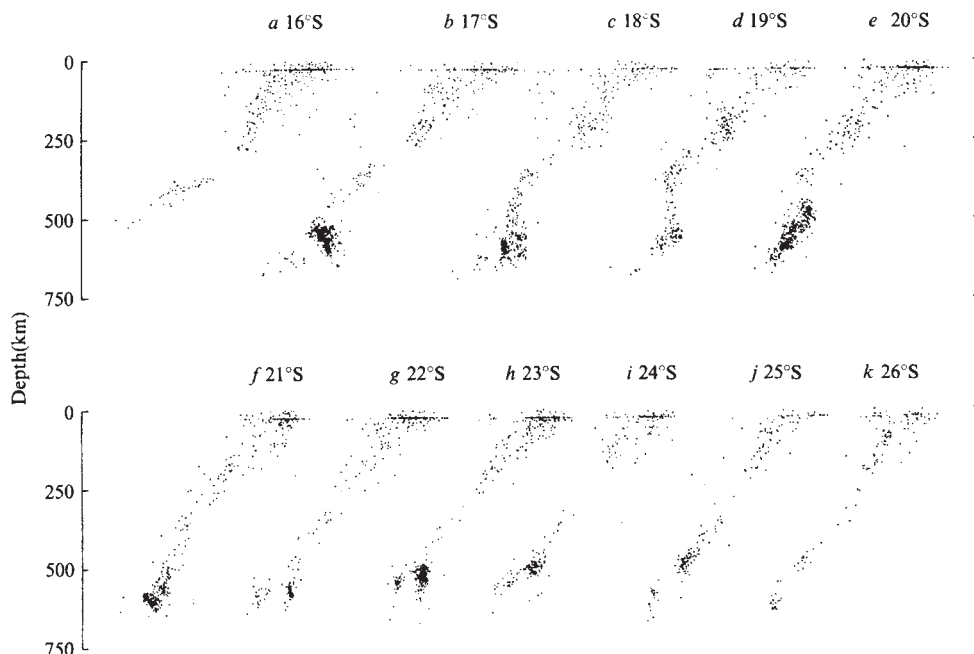


Fig. 2 Vertical sections through the Tonga Benioff zone between 16 and 27° S. The sections are along parallels of latitude, viewed from the south. Each is 1° thick and includes seismicity to the south of the indicated parallel.

approximate agreement with one, or occasionally both of the nodal planes; from this we infer the existence of planes of shear failure. The complex pattern of shear zones identified by this technique enables us to begin to elucidate the structural geology of the subduction zone.

Morphology

The Tonga and New Hebrides subduction zones form a highly complex part of the Melanesian boundary between the Indian-Australian plate and the Pacific plate (Fig. 1). The Benioff zone studied here is associated with the subduction of the Pacific plate to the west beneath the Indian-Australian plate and both the characteristics of the seismicity³¹⁻³⁵ and the nature of stress release in the subducting slab^{6,17,35-37} have been studied. The inclined seismic zone terminates in the characteristic 'hook' shaped region of intense deep activity. All of the CMT events analysed in this study are located within this 'hook' (Fig. 4).

Figure 2 shows a sequence of vertical sections through the Benioff zone, having a thickness of 1° in latitude. Notable here is the extreme distortion and variable geometry of the zone. The separation of the seismicity into double seismic features, at great depth, is evident in several sections (Figs 1, 2*b*, *c*, *g-j*). Figure 3 shows a projection onto a vertical meridional plane, looking from the west, and displays some aspects of the vertical distribution of events.

For our later discussion we identify four principal regions within the zone of deep activity (see Fig. 3):

The northern region (17°–19° S): Intense seismic activity at the northern end of the 'hook' forms an inclined column. Its vertical extent can be seen in Fig. 3. A weak secondary line of activity flanks the region to the south-west (Fig. 1).

The central region (19°–21.5° S): This is a region of diffuse seismicity dipping at a shallower angle than other parts of the deep seismic zone (Figs 2*d-f*, 3).

The southern region (21.5°–27° S): This region is characterized by two parallel bands of seismicity (Fig. 1). The weaker band lies to the west and penetrates to greater depths, particularly in the south (Fig. 2*g-k*).

The gap (22.3°–23.2° S): The principal band of deep seismicity is interrupted by a distinct gap (Figs 1, 3). This gap appears to correspond with the gap in shallow activity (Fig. 1) which separates the Tonga and Kermadec Trenches, and coincides with the intersection of the Louisville Ridge with the trench at ~25° S.

Moment tensor solutions

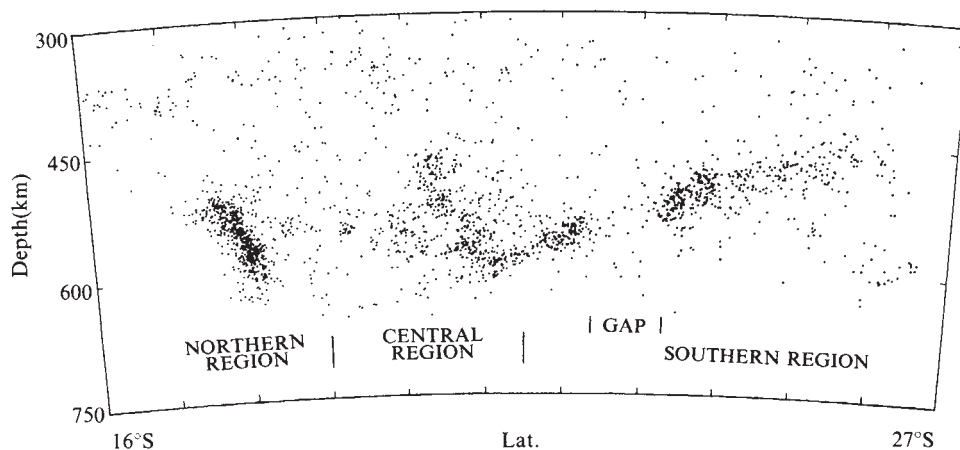
We shall discuss here a set of 17 moment tensor solutions and their implications for deformation within the subduction zone. Many of these are representative of families of solutions showing the same behaviour. The moment tensor is a second rank symmetric tensor, which may be characterized in terms of its principal axes and its eigenvalues. The moment tensors determined in this study are constrained to have zero trace, corresponding to a source without volume change, and thus the sum of the eigenvalues is zero. A positive eigenvalue represents extension at the source and a negative eigenvalue corresponds to shortening. The largest eigenvalue is positive and is associated with the tension axis (T-axis) and the smallest eigenvalue is negative and is associated with the compression axis (P-axis). The intermediate eigenvalue is smallest in absolute value. In agreement with previous studies^{6,17,35} we find that in Tonga the P-axis is usually in the down-dip direction, indicating net shortening of the zone. This can be seen in Fig. 4, where the P-axis is indicated by '+'. The moment tensor solutions are represented here in terms of an equal area projection of the lower focal hemisphere. The intermediate axis is at the intersection of black and white quadrants.

A source represented by a shear dislocation has a vanishing intermediate eigenvalue and P and T eigenvalues of equal magnitude, with opposite signs. Such a source is said to have a 'double couple' mechanism. We find that in Tonga the compressive eigenvalue tends to be the largest in absolute value (though other subduction zones exhibit different behaviour^{26,38}). Here, however, we shall neglect this departure from the condition for plane shear failure, and concentrate on the geometry of the 'best double couple' solution.

The planes separating black from white in Fig. 4 are orthogonal nodal planes, which intersect in the intermediate axis and are inclined at 45° to the other two axes. The best double couple solution may be interpreted in terms of shear failure on either of these planes, in which relative motion is perpendicular to the intermediate axis and is in the sense which would take a material particle from a white into a black quadrant.

In Fig. 5 we have taken, as the centre of our field of view, events for which CMT solutions have been found, and displayed the local pattern of seismicity as seen from the direction of the intermediate axis. From this direction any lineations in the pattern of seismicity associated with planes of shear failure should be apparent, and should be aligned with one of the nodal

Fig. 3 Lateral projection of the seismicity on a vertical meridional plane viewed from the west. The subdivisions correspond to subregions of the Benioff zone identified in the text.



planes. We find that such lineations are nearly always discernible in the seismicity patterns of the relocated events, and we interpret these lineations as zones of shear failure. Viewed from the direction of the intermediate axis the nodal planes appear as straight lines which divide the circle into quadrants. The direction of maximum shortening (the P-axis) lies in the white quadrants and the T-axis lies in the blackened quadrants. These are orthogonal and are inclined at 45° to the nodal planes.

In each part of Fig. 5 the latitude, longitude and moment of the event are indicated, together with the direction of view and the scale of the box. Figure 5a, for instance, is centred on an event of 21 January 1977 (moment, 10^{25} dyn-cm), viewed from an azimuth of 158° , with the line of sight inclined slightly upwards, at an angle of 3° to the horizontal. The sides of the box are of length 300 km and the depth of the box in the direction of the line of sight is 160 km. A set of criteria on size, reliability of location, and azimuthal coverage of stations has been specified in each part of Fig. 5 to clarify the feature which we believe to be associated with the CMT event. In a few cases it has proved difficult to separate such features from the background seismicity which presumably is indicative of other modes of deformation; in such cases the time sequence of events has often convinced us of the reality of the lineation.

Northern region

Figure 5a–c shows events located in the northern region (Figs 3, 4). In Fig. 5a we would identify the approximately vertical lineation as a plane of shear failure. Figure 5b shows the same cluster of events seen from a steep angle. The events on the right in Fig. 5c are coincident with some of the events of Fig. 5a, but here the CMT event lies in the weak secondary zone of seismicity lying to the south-west (Fig. 4). In this case there are rather few events to define the lineation; the pattern is very similar, however, to the patterns observed in southern Tonga to be discussed below (Fig. 5l–r).

Central region

CMT events in the central region (Figs 3, 4) may be classified into two distinct families. Figure 5d–f shows representatives of the first family, and indicates a shearing deformation approximately in the plane of the Benioff zone. The second family (Fig. 5h, i) shows out-of-plane shear. Events in the first family (Fig. 5d–f) all show motion in the same sense within three subhorizontal lineations, gently dipping to the north and located at different depths (event in Fig. 5f is located in the lowermost lineation). This indicates a mode of deformation of subhorizontal shearing of the zone. A cross-cutting conjugate lineation is also apparent (Fig. 5d). The events of Fig. 5f, g show motion on different planes which are internal to the lowermost shear zone and are indicative of an organized internal structure. Events of the second family are shown in Fig. 5h, i. A complex structure of subhorizontal planes dipping at $\sim 30^\circ$ to the north-west becomes apparent.

We infer that in this central zone there are simultaneously active modes of shear deformation both in and out of the plane of the Benioff zone. Both the moment tensor solutions and the seismicity patterns indicate thickening and bottoming out of the descending material, and suggest that subduction experiences great resistance in penetrating the 670-km discontinuity.

As we have noted above, the gap in shallow seismicity which separates the Tonga subduction zone from the Kermadec zone is associated with the intersection of the Louisville Ridge with the trench and this gap appears to correspond to the gap in deep seismicity (Figs 1, 3). If, however, the ridge were embedded in a rigid subducting slab it would intersect the deep seismic activity much farther to the north. It is possible to identify the gap in deep activity with the Louisville Ridge if the entire zone

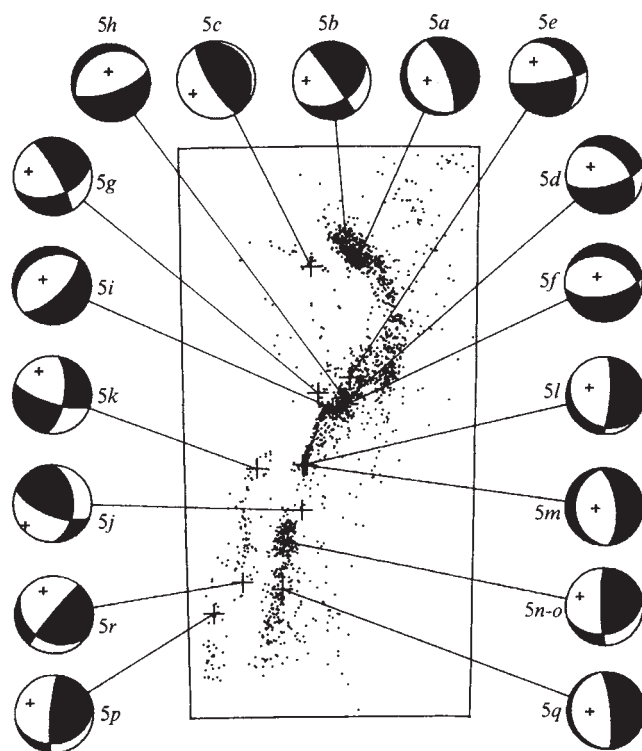
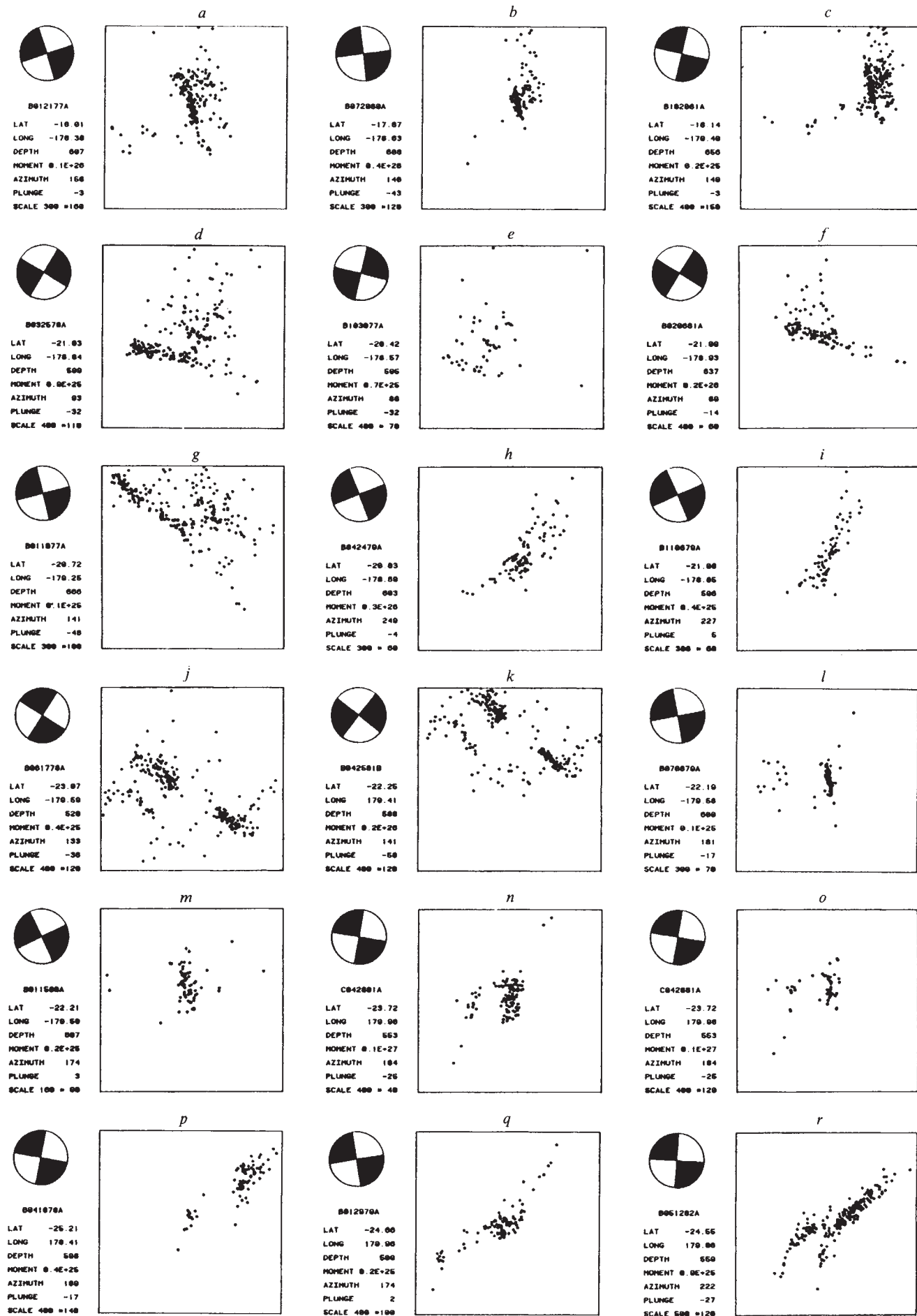


Fig. 4 Focal mechanisms of the selected CMT solutions. For each event the equal area projection of the lower focal hemisphere is shown, viewed from above; '+' represents the compressional axis and the black sectors indicate extension at the source. Relocated seismicity is plotted, deeper than 300 km and with latitude between 27° and 16° S and longitude between 178° E and 176° W. Each CMT event is identified by a number corresponding to events detailed in Fig. 5.



has suffered horizontal shearing in which deeper material moves relatively south. This is the same sense as the shear inferred from Fig. 5d-f.

The gap

Figure 5j, k shows two events associated with the gap in deep activity. These are anomalous in that they do not show down-dip shortening and are of opposite sense. We note, however, that they are on opposite sides of the gap, and are both consistent with motion of the material in the gap, approximately in the down-dip direction, at a faster rate than the surrounding material.

Southern region

Figure 5l-r shows events in the southern region, both to the north (Fig. 5l, m) and to the south (Fig. 5n-r) of the gap. The almost vertical lineation is very clear in Fig. 5l; this lineation indicates a backward bending of the deepest part of the descending flow (see Fig. 2g). Figure 5m shows another event in the same cluster; the enlarged scale reveals a pattern of approximately conjugate alignments.

Figure 5n-r shows events associated with the double seismic feature in the south. Figure 5n, o shows the same event; in Fig. 5n a very thin slice has been taken and in Fig. 5o only larger events are shown. The seismic zone is very narrow for large events and the more diffuse pattern of Fig. 5n appears to be associated with the occurrence of smaller events on conjugate planes (see Fig. 2g, h). The vertical alignments in Fig. 5n-o, r indicate shear deformation in which material to the east moves relatively down. The event shown in Fig. 5p is in the secondary region of seismicity and shows motion in the same sense. The separated double seismic zone is interrupted by a connecting 'bridge' of seismicity at $\sim 25^\circ$ S (see Fig. 2i). Figure 5q shows an event located in this 'bridge', and here both horizontal and vertical alignments can be seen. The pattern of parallel, subvertical shear zones in which the sense of motion is such that trenchward material moves relatively down is reproduced by other solutions not presented here. Similar behaviour in the northern region has been noted above (Fig. 5a-c).

Figure 5r, which we interpret as shear on the central vertical plane, also shows this behaviour and appears to present us with a snapshot of the slab breaking and being displaced sideways as it reaches the bottom of its trajectory. The double seismic feature at the base of the Tonga subduction zone would represent, in this case, the debris of slab unable to penetrate deeper into the mantle.

Discussion

The correlations we have shown between the orientation of the nodal planes of deep earthquakes and the local pattern of seismicity can be found only if the nodal plane is known very precisely. Accurate hypocentral locations are also necessary, but in fact all of the features we have shown are identifiable using published ISC coordinates; the relocation of events serves principally to bring the patterns into better focus.

This analysis enables us to understand the apparently accidental orientations of the nodal planes in terms of fairly well defined modes of deformation—vertical shear in the northern and southern regions, and subhorizontal shear in the more diffuse

central region. In each case shortening occurs in the down-dip direction and material is displaced laterally.

The concept of a subduction zone as a smoothly bending, coherent slab, possessing a uniform thickness defined by the seismicity is in need of modification. When seismicity is analysed in detail, we see a complex, cross-cutting pattern of interacting 'shear bands', and we are led, rather, to view the Benioff zone as that part of the convective flow which, by virtue of temperature, composition and strain rate accomplishes its deformation through episodes of shear instability. Viewed on a time scale of several million years the zone would appear to deform smoothly—and perhaps to no lesser extent than the surrounding mantle.

If, over geological time, the nodal planes were randomly oriented with respect to the P-axis, the result would be thickening and shortening without distortion. If, on the other hand, certain orientations remain dominant over long periods, they may lead to distortion and separation of parts of the slab such as we see in Tonga.

The transition to horizontal shear or to lateral separation in the deepest part of the seismic zone is evidence of the strong resistance experienced by the subducted material to penetration of the 670 km discontinuity. We observe horizontal displacement of the seismically active portion of the slab, and infer that material moves laterally rather than vertically at the base of the deep seismic zone. This favours the view that subduction does not continue into the lower mantle, although we clearly cannot infer the fate of the subducted material after it ceases to be seismically active.

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Fig. 5 (Opposite) Each part shows the CMT solution and the associated seismicity viewed from the direction of the intermediate axis; the CMT event is located in the centre of the box. The hypocentral position and the scalar moment are indicated for each solution. The azimuth and plunge identify the direction of the intermediate axis. The scale indicates the lateral dimension and thickness of the box of plotted seismicity, both in km. The nodal planes appear as perpendicular lines which divide the focal hemisphere into quadrants of compression (white) and extension (black) at the source. The compressional axis is located in the white quadrants, at 45° to the nodal planes. Only relocated seismicity is shown.

Nd–Sr isotopic composition of granulites and constraints on the evolution of the lower continental crust

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In a statistical study of granulite facies rocks, we have analysed 32 samples with ages ranging from 2,900 to 200 Myr for Sm, Nd, Rb and Sr and $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. The Sm/Nd ratio is found to be constant compared with the highly variable Rb/Sr ratio. Using the model age formalism, we show that, in many cases internal differentiation of continental crust follows external differentiation with a large time interval.

SEISMIC studies of the continental crust show that the lower continental crust has quite specific physical properties. From geological observations and studies of drilled samples it has been established that the upper continental crust is composed of sedimentary rocks which overlie a basement mainly composed of metamorphic and granitic rocks. The nature of the lower crust is more controversial. The seismic velocities of lower crustal levels (ranging from 6.6 to 7.2 km s⁻¹)¹ correspond to those measured for metamorphic and igneous rocks equilibrated in the granulite facies. Indeed, high pressure experiments show that such metamorphic assemblages are generated in pressure–temperature conditions similar to those prevailing in the lower continental crust. These observations have led several authors (for example refs 2–9) to abandon the old concept of a lower basaltic crust and to consider a lower crust mainly composed of granulites (in the sense of granulite facies rocks). Such granulites are mixtures of meta-sedimentary and meta-igneous (acid and basic) rocks, both metamorphosed in the same (high *P*, high *T*) conditions.

Heat flow interpretations in continental environments and direct measurements of U, Th and K concentrations in granulites have led several authors^{10–13} following Heier⁴ to postulate that the lower crust is depleted in U, Th, K and Rb. Such a depletion would explain the enrichment of the upper crust in these elements. According to these authors, this depletion would result from a general differentiation into an upper and a lower level within the continental crust in association with crustal anatexis and/or fluid migration; this would represent a fundamental process of continental crust evolution.

Thus, when considering the formation and development of the continental crust one must distinguish two potentially distinct geological processes: first the process by which a piece of new continental crust differentiates at the expense of the mantle (referred to as external differentiation of continental crust or EXCOD) and second, the process by which the upper and lower levels differentiate within the continental crust (referred to as internal differentiation of the continental crust or INCOD). What are the exact relationships between EXCOD and INCOD? Do they occur simultaneously or independently in time? What are their relationships with the orogenic processes? Are the answers to those questions unique? Any contribution to these problems may be critical to solving our understanding of the development of the continental crust.

Recently¹⁴, the genesis of upper continental crust was investigated on a large scale through the study of granitoid rocks of different ages, using the coupled Sr and Nd isotopic tracers. The main result of this statistical study was that, in general, the Precambrian granitoids have a signature relatively close to that of the mantle whereas clear evidence was found for recycling processes being involved in the genesis of young granitoids. Although this approach was based on a limited number of

samples, it has been confirmed by several case studies of granitoid rocks throughout the world: western US^{15,16}, Caledonian¹⁷ and Hercynian¹⁸ granites in Europe, Namibian of Africa¹⁹, Western Australia²⁰. The genesis of old granitoids is not so simple because the Nd²¹ and Sr isotopic growth curves of the Archaean mantle are still debated and because lead isotope studies suggest that the genesis of old granitoids is similar to that of young ones²².

Using a similar approach for the lower continental crust, the present study surveys various types of granulites of different geological ages. It will be followed by some detailed case studies.

The study of granulites is more complex than that of granitoids because they are not direct igneous products with a well defined age of intrusion. They rather frequently correspond to polymetamorphic assemblages with quite complex tectonometamorphic histories; some were originally igneous rocks, some others were sediments, as testified by the presence of abundant marbles in granulitic formations. Therefore isotopic results in granulite terranes generally require detailed discussion, integrating geological constraints.

In a strict petrological sense, granulite facies rocks correspond to regional high grade metamorphism with rare hydrous minerals, high rock densities (3 g cm⁻³) and high temperature–high pressure paragenesis. Common features are the presence of hypersthene and clinopyroxene in calcic assemblages, the presence of myrmekite, greyish blue quartz and pleochroic hypersthene in acidic assemblages, with plagioclase and K-feldspar.

We have selected several samples of various ages on the criterion that they correspond to an unambiguous definition of the granulite facies (as quoted above) for which different geological and petrological studies (done previously) yield independent information on their history. The largest part of our samples were obtained through the courtesy of P. Hurley and were mainly studied by Spooner²³, and Spooner and Fairbairn²⁴. These are acid and intermediate varieties of pyroxene granulites following the definition of Spooner²³ (instead of 'charnockite' according to Holland's definition²⁵). The other samples come from In'Ouzzal (Ahaggar), Ansignan and Agly (Pyrenees), from several different areas in Norway, and also include xenoliths from Le Bournac (France), kinzigites from Beni Bousera (Moroccan Rif) and the Ivrea zone (Italian Alps). In each case, the petrographic description can be found in the literature referenced with a brief description of each area in the caption of Fig. 4.

Chemistry of granulite rocks

For each sample we have determined the Nd isotopic composition and the Sm–Nd concentrations through isotope dilution. For some samples, we have determined the Rb–Sr concentra-

tions and Sr isotopic compositions, while in some others, we have used the values published by Spooner and Fairbairn²⁴. The analytical techniques used have been described in detail elsewhere^{14,26,27}, but we wish to emphasize the particular point of zircon dissolution. For every sample, after dissolution in an open Teflon beaker, heavy minerals are dissolved in a closed, pressured Teflon bomb at 205 °C. Indeed, since heavy minerals contain high proportions of rare earth elements (REE)²⁸, such a technique is crucial for granulites as well as for granulites. Some workers either do not use bomb dissolution or do not give any indication about their analytical procedure^{29–35}. All the corresponding results are dubious and should be used with caution. However, due to a lack of enough reliable analyses in literature, we have used these results in our statistics. However, this incomplete dissolution of zircons may enlarge the real spread of the $^{147}\text{Sm}/^{144}\text{Nd}$ and the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios. All the experimental results are reported in Table 1, with error limits.

Our selection represents only 32 specimens of typical granulites and therefore cannot be considered as a fully representative worldwide sample of this facies. However, a comparison between these results and those obtained on granulites as referred to lower and upper continental crust respectively is of great interest.

In relation to petrological variations within the granulites, Nd and Sm concentrations vary from 3 to 65 p.p.m. and 0.4 to 11 p.p.m. respectively (Table 1). The average values of 28.2 p.p.m. for Nd and 5.3 p.p.m. for Sm are relatively close to those obtained for the upper continental crust and comparable with the average values of the main components of the North American continental crust: for shales, Nd = 31 p.p.m.; for the Precambrian shield composite (north Quebec + Saskatchewan + south-west Quebec) Nd = 30.7 p.p.m. More significant is the relative clustering of the $^{147}\text{Sm}/^{144}\text{Nd}$ ratios at around 0.121 (Fig. 1a). The average value for granulite rock is around 0.113 and is close to the value for granulites (Fig. 1b). In addition, the average value for the whole crust estimated by De Paolo and Wasserburg³⁶ is 0.116 and estimated by Jacobsen and Wasserburg³⁷ is 0.119, which are both remarkably close to our average. However, the $^{147}\text{Sm}/^{144}\text{Nd}$ ratios in the continental crust are not strictly constant since the measured values vary from 0.06 to 0.20 in granulites and from 0.07 to 0.16 in granulites. When considering basic ortho-derived granulites and xenoliths, the spread largely overlaps the mid-ocean ridge basalt (MORB) field. However, from a statistical point of view, the distinction between acidic continental material and MORB (Fig. 1c) is remarkable.

Both the absolute contents and the ratios of Rb and Sr are highly variable (Table 1). Rb and Sr contents vary from 2.4 to 117 p.p.m. (a factor of 49), and from 31 to 870 p.p.m. (a factor of 28) respectively; Sr varies more than the REE. $^{87}\text{Rb}/^{86}\text{Sr}$ ratio varies from 0.09 to 4.8 (a factor of 55). We emphasize that all values are equal to or larger than the bulk Earth value which is around 0.09. Therefore there is no doubt that the bulk continental crust (lower + upper crust) has a higher Rb/Sr ratio than that of the bulk earth.

Amongst several authors, Heier⁴ claimed that rocks of the granulite facies are strongly depleted in Rb relative to Sr. This is true of the Lewisian granulites^{38,39} and a few others²⁴ but does not appear to be a general rule as shown by Leyreloup *et al.*⁴⁰, Allègre *et al.*⁴¹ for Bournac granulites and by Spooner and Fairbairn's²⁴ extensive study. Available Rb/Sr ratios data for granulites are shown in Fig. 2. The observed scatter indicates that some areas are depleted in Rb whereas others are not. The cause of this difference is not understood but must be taken as a fact.

The raw data for the $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ isotopic ratios (Table 1), uncorrected for age, have been directly reported on the Sr–Nd correlation diagram (Fig. 3). In addition, we have plotted the field defined for granulites taken from the literature. In their study of Lewisian granulites, O'Nions' group^{33,42} have defined a field for granulite with low $^{87}\text{Sr}/^{86}\text{Sr}$ and negative ϵ_{Nd} . However, many granulite-facies rocks plot outside this field. In

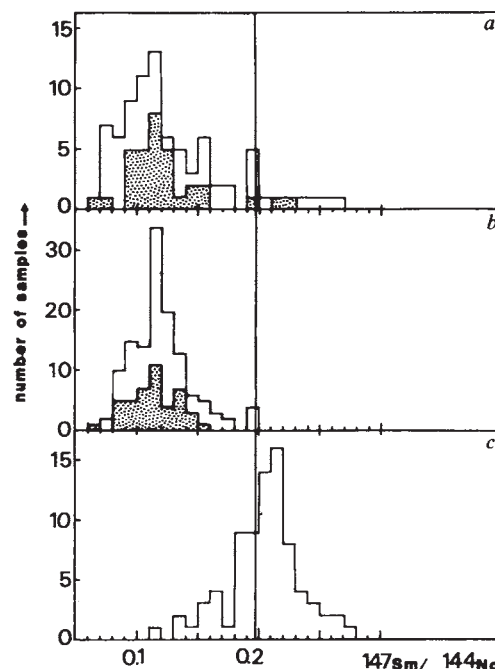


Fig. 1 Histograms of the $^{147}\text{Sm}/^{144}\text{Nd}$ ratios. a, In granulites (data (■) are from this paper, the others (□) are from refs 29, 30, 32–35). b, In granulitoids (data (■) are from refs 14, 20, 52, 74, the others (□) are from 15–19). c, In MORB.

fact, the field for all the granulites would largely overlap that for granulitoids.

Without discussing the detailed age relationships and considering only the geological age we must point out that, as for granulites, ϵ_{Nd} values are mainly a function of the rock ages due to fairly constant Sm/Nd ratios of the continental crust. On the other hand, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratios are more variable but the scatter seems to increase with age. This also reflects the great variability of Rb/Sr ratios.

Model age systematics

The results will now be discussed in terms of model ages of our samples, as defined by McCulloch and Wasserburg³¹. Since continental crust material ultimately derives from the mantle, a model age relative to their mantle source will be estimated. In doing so, the evolution curve of the depleted mantle, as defined by MORB data will be chosen^{14,15}. The details of the calculations are given in Table 1 legend.

As the Sm/Nd ratio of the continental crust is fairly constant, the Nd model age is expected to be 'robust' relative to a complex evolution of the continental crust. In contrast the Rb/Sr fractionation history of the crust prevents Sr model age from being geologically significant. A comparison of both model ages will be very useful in deciphering the history of each particular segment of the continental crust.

For each area we have reported the radiometrically determined geological ages of the granulites. In most cases these ages are obtained by whole rock Rb–Sr (often poorly defined) isochrons (Liberia, Tanzania, Columbia, Venezuela, Bournac, Norway, Rogaland and Beni Bousera). In some cases U–Pb ages are available from zircon studies (In'Ouzal, Amitsoq, Bournac, Ivrea zone, Venezuela). Last, in a few cases, ages have been obtained by whole rock Pb–Pb dating (Venezuela).

Because the Rb–Sr and U–Pb systems are often perturbed by metamorphic events, ages must be interpreted with caution. When a granulite is ortho-derived, the meaning of its age is clear; it probably represents the time of intrusion of its progenitor. When the rock is para-derived, the meaning is more conjectural. Does it correspond to a large-scale rehomogenization contemporaneous with an orogeny⁴³, or to mixing or the

Table 1 Analysis of typical granulites

Samples		Nd	Sm	$^{147}\text{Sm}/^{144}\text{Nd}$	$^{143}\text{Nd}/^{144}\text{Nd}$	Rb	Sr	$^{87}\text{Rb}/^{86}\text{Sr}$	$^{87}\text{Sr}/^{86}\text{Sr}$	ϵ_{Nd}^0	ϵ_{Sr}^0	Nd Model ages (Myr)	Geological ages (Myr)	Metamorphism ages	Sr model ages
Beni Bousera															
BB6*	Kinzigit	28.3	5.35	0.113	0.51206±3	76.4	183.4	1.19	0.72259±16	-10.9	253	1,493	20	20	1,200
BB9*	Kinzigit	31.7	6.35	0.120	0.51198±3	77.7	128.8	1.72	0.72445±11	-12.5	279	1,737	20	20	900
BB10*	Kinzigit	36.2	6.54	0.108	0.51199±3	139.6	208.6	1.91	0.72468±10	-12.3	282	1,524	20	20	818
BB12*	Kinzigit	35.9	6.37	0.106	0.51206±5	44.2	222.2	0.575	0.71958±18	-10.9	210	1,397	20	20	2,198
M6 482*	Kinzigit	23.8	4.65	0.117	0.51206±3	165.2	229.5	2.05	0.72120±30	-10.9	233	1,555	20	20	640
Ivrea zone															
ST 8*	Acid granulite	24.9	5.17	0.125	0.51237±5	32.9	228.4	0.417	0.713521±31	-4.9	124	1,164	480	260	1,978
ST 10*	Basic granulite	15.7	3.68	0.142	0.51226±4	4.45	248.0	0.052	0.71014±40	-7.0	76	1,672	480	260	—
ST 13*	Kinzigit	11.8	3.08	0.158	0.51237±3	101.3	105.0	2.79	0.726183±17	-4.9	304	1,854	480	250	596
AGLY 1	Leptinitic gneiss	16.869	4.017	0.1432	0.51232±4	117‡	328‡	1.048‡	0.73911‡	-5.8	487	1,569	553	300	2,539
Norway															
NOR 15	Larvikite	45.138	9.632	0.1290	0.51240±4	91.3‡	183.4‡	1.44‡	0.7256±1*	-4.3	289	1,165	1,000	1,000	1,137
NOR 73	Arendalite	14.192	4.990	0.2126	0.51271±4					1.7		—	1,000	1,000	
NOR Rog. 65/75	Charnockite	31.117	4.903	0.0953	0.51191±4					-13.8		1,464	1,000	1,000	
39/73		48.427	8.658	0.1081	0.51118±35					-28.1		2,732	1,000	1,000	
Lapland	Metagabbro	24.10	5.13	0.1290	0.51221±3	39‡	263‡	0.428‡	0.71638±7‡	-8.0	165	1,505	1,000	1,000	2,466
Le Bournac 801	Paragneiss	36.69	7.011	0.1156	0.51210±2			0.563‡	0.7131‡	-10.1		1,471	1,000	380	1,360
802	Orthogneiss	44.726	6.744	0.0915	0.51237±2					-4.9	118	851	1,000-200	380	
Bal 9	Paragneiss	65.03	11.512	0.1071	0.51197±3					-12.6		1,540	1,000-200	380	
Bal 142	Orthogneiss	52.99	9.899	0.1129	0.51223±2			1.63‡	0.7274‡	-7.6	320	1,236	1,000-200	380	1,081
Columbia 7276		41.32	7.984	0.1169	0.51213±2	210	124	4.814	0.8027	-9.6	1,389	1,443	1,270-1,370	1,200	1,471
Tanzania 7051	Charnockite	8.62	1.815	0.1260	0.51131±2	71‡	410‡	0.459‡	0.72118±6‡	-25.6	268	3,090	2,232-2,692	2,600	3,143
						67	427	0.504	0.7237		232				3,228
Uganda 7012	Aplitic granulite	2.970	0.390	0.0794	0.51057±5	135	31	13.241	1.1969	-40	6,982	2,848	2,600		2,636
7020	Aplitic granulite	15.582	2.999	0.1164	0.51077±4	207	78	8.003	1.0081	-36.1	4,304	3,688	2,600		2,701
India 7217	Basic charnockite	4.993	1.611	0.1957		2.4‡	80‡	0.087‡	0.7045±6‡				2,563	2,500-2,400	2,684
								0.147	0.7077						3,650
7246	Charnockite	13.369	3.478	0.1572	0.51206±3	14.5	201	0.208	0.70910±5‡	-10.9	61	2,723	2,560	2,500-2,400	2,678
Liberia NR 6164		18.014	2.910	0.0977	0.51028±3	88.6‡	272‡	0.952‡	0.74643±4‡	-45.6	591	3,744	2,642-2,692	2,700	3,406
GCM 6224		22.318	4.424	0.1199	0.51106±4	62.4‡	209‡	0.871‡	0.73936±3‡	-30.4	491	3,315	2,596-2,643	2,700	3,124
Venezuela 8100A2					0.51081±1					-35.3		2,800		2,000	
		15.862	2.479	0.0941	0.51087±8	74.5	100.6	2.162	0.7968	-34.1	1,305		2,020-2,800	2,000	3,130
					0.51077±4					-36.1				2,000	
Guyana 7494		33.225	3.817	0.0695	0.51060±1				0.70857±4‡	-39.4	618	2,611	2,020-2,800	2,000	
8204		65.663	10.751	0.0990	0.51140±2	67.1‡	870‡	0.225‡	0.70854±3‡	-23.8	54	2,201	1,975-2,133	2,000-2,100	2,150
In'Ouzzal M408	Charnockite	6.274	1.454	0.1391	0.51156±7					-20.7		3,123	3,100-Pb	2,100	
L101	Charnockite	39.471	7.668	0.1176	0.51092±6	73‡	55.7‡	3.90‡	0.870‡	-33.2	2,344	3,473	2,946-Sr	2,100	3,055
Surinam 8322				0.108	0.51146±2			0.088	0.7048	-22.6	0.28	2,305	2,055-2,760	2,000-2,760	

The Rb-Sr analyses are done either by D. Rousseau (‡), J. Hamet (†) or M. Polvé (*), the others are done by the MIT Group (see references). Blanks for the Rb and Sr analyses done either by D. Rousseau, J. Hamet or M. Polvé are around 70 pg and 160 pg respectively. Blanks for Nd and Sm done either by D. Ben Othman or M. Polvé, are around 100 pg and 70 pg respectively. $^{87}\text{Sr}/^{86}\text{Sr}$ ratios are normalized using $^{86}\text{Sr}/^{88}\text{Sr}=0.1194$; errors are quoted at $2\sigma_m$. Nd is run as Nd+. $^{143}\text{Nd}/^{144}\text{Nd}$ ratios are normalized to $^{146}\text{Nd}/^{144}\text{Nd}=0.7219$, errors are quoted as $2\sigma_m$. The average measured value of Nd standards from Johnson Matthey is 0.511981 ± 0.000015 . Nd model ages are given by the intercept between two curves: $(^{143}\text{Nd}/^{144}\text{Nd})_{\text{sample}} = (^{143}\text{Nd}/^{144}\text{Nd})_T + (^{147}\text{Sm}/^{144}\text{Nd})_{\text{sample}}(e^{A'T} - 1)$ and $(^{143}\text{Nd}/^{144}\text{Nd})_{DM} = A'T^2 + BT + C$. DM: Depleted mantle, with $A = 1.53077 \times 10^{-5}$; $B = -0.22073 \times 6.54 \times 10^{-5}$; $C = 0.513078$. A, B, C are chosen to fit the evolution curve of a depleted mantle defined by data on MORB, ophiolites (Bay of Island, Semail), Precambrian mafic volcanics (Kambalda, Kanowna, Onverwacht), komatiites (Cape Smith, Abitibi), Isua supracrustals and meteorites (Angra dos Reis, Juvinas)⁶⁴⁻⁷¹. Sr model ages are given by the intercept between two curves $(^{87}\text{Sr}/^{86}\text{Sr})_{\text{sample}} = (^{87}\text{Sr}/^{86}\text{Sr})_T + (^{87}\text{Sr}/^{86}\text{Sr})_{\text{sample}}(e^{A'T} - 1)$ and $(^{87}\text{Sr}/^{86}\text{Sr})_{DM} = A'T^2 + B'T + C'$ with $A' = -1.54985776 \times 10^{-4}$; $B' = -1.6007234 \times 10^{-4}$; $C' = 0.70273029$. A', B', C' are chosen to fit the evolution curve of a depleted mantle defined as for Nd by data on MORB, ophiolites (Zambales, Toba, Guatemala, Bay of Island), komatiites (Cape Smith, Abitibi), BABI and Allende^{64,69,72,73}.

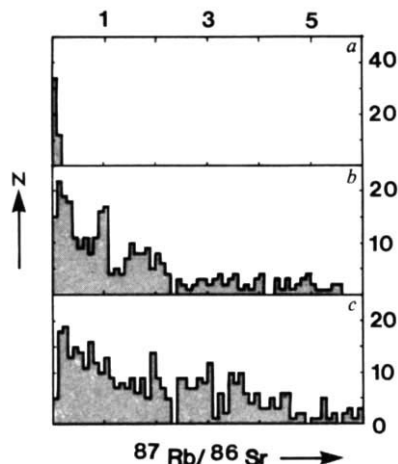


Fig. 2 Histograms of the $^{87}\text{Rb}/^{86}\text{Sr}$ ratios. *a*, In granulites (data are from this paper and refs 11–13, 23, 24, 34, 38–40, 45, 47, 49, 54, 63, 75–88 and J. Hamet and C.J.A., in preparation). *b*, In granulitoids (data are from refs 14, 16, 17, 19, 20, 53, 54, 74, 89–99). *c*, In MORB.

age of the sediment sources? In the case where the granulitoids present in the same region have the same age, this age is most probably related to a regional episodic event. We will call this age the geological age. Aware of these possible complications, we have plotted the geological age versus the Nd model ages in Fig. 4a for the granulites. Additional data are for Lewisian gneisses³³, Lofoten, Norway³⁰, Australian samples³⁴ and Antarctic²⁹.

As can be seen, the Nd model ages are in most cases greater than the geological ages. This is especially true for the younger samples: Bournac, Norway, Ivrea zone, Beni Bousera, and for the older African gneisses (Liberian, Uganda, Tanzania, In'Ouzzal).

For some older samples such as those from India, Australia, Columbia, Guyana and Scotland, the Nd model age is close to the 'geological age'. For two samples from Venezuela, the 'geological age' is greater but is also poorly determined.

In several cases, it is possible to determine with reasonable accuracy the time at which the rocks were metamorphosed to granulite facies. The geological significance of this event is more clear than the previously discussed geological ages. This is the case for samples from In'Ouzzal (Ahaggar), Imataca (Venezuela), Lofoten and Bamble (Norway), Bournac (France), Ansignan (France), Beni Bousera (Morocco), the Ivrea zone (Italy), and the Lewisian gneisses (Scotland).

The garnet-whole rock Sm–Nd age for In'Ouzzal (D.B.O. and C.J.A., in preparation), the zircons of some metasedimentary rocks⁴⁴ and petrological studies all give an age of 2,100 Myr which also corresponds to the age of the Tassendjanet pluton and of a main orogenic episode in this area.

In the Imataca area (Venezuela) studied by Hurley *et al.*^{45,46} and Gaudette *et al.*⁴⁷ granulite-facies metamorphism occurred at 2,000 Myr which also corresponds to an important tectono-metamorphic event of this area.

The Lewisian complex studied by Hamilton *et al.*³³ yields an age of about 2,600 Myr for granulite-facies metamorphism which is the age of a major orogenic event associated with granulite injection.

The Lofoten occurrence in Norway studied by Jacobsen and Wasserburg³⁰ gives an age of 1,700 Myr for granulite facies metamorphism. This age corresponds in Norway to a major tectono-metamorphic event with associated plutonism.

In the Bamble area, also in Norway, the granulite facies metamorphism is dated at 1,000 Myr, an age which also corresponds to the major tectono-metamorphic event in this area, again associated with important plutonism^{49–51}.

In the Bournac case, a Sm–Nd garnet whole-rock isochron⁵² gives an age of 380 Myr apparently linked to the end of the

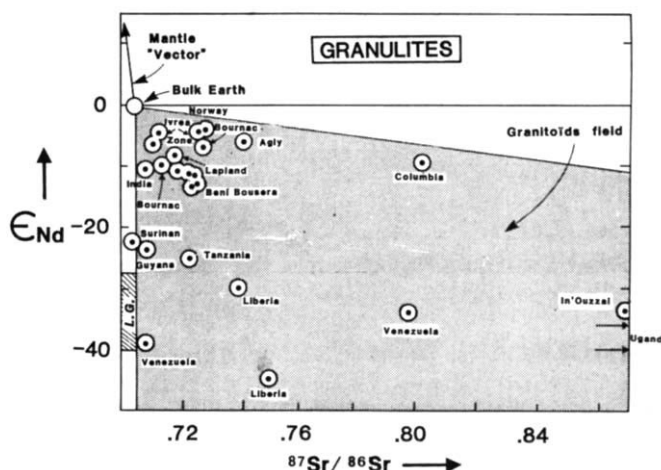


Fig. 3 Data from granulites plotted in the ϵ_{Nd} versus $^{87}\text{Sr}/^{86}\text{Sr}$ diagram in comparison with the fields of granitoids and Lewisian gneisses.

Caledonian orogeny and slightly older than the intrusion of the Velay granite, the latter being 300 Myr old⁵³.

The Agly granulite was formed 300 Myr ago during the Hercynian orogeny and is associated with granulite intrusions such as Querigut and Milhas⁵⁴.

The Ivrea zone granulites are 260 Myr old (M. Polvé and C.J.A., in preparation), the same age as the granitic intrusions in the neighbouring amphibolite facies zone^{55,56}.

The Beni Bousera granulite-facies metamorphism is quite young (20 Myr) (M. Polvé and C.J.A., in preparation). Such a metamorphic event seems to be linked with the regional tectonics^{57,58}. However, no important plutonism is presently known in the area.

Comparison of these ages for granulite metamorphism with the Nd model age clearly shows that they are generally much younger. This information is almost the same as the one given by the geological age. The Venezuela case seems to be an exception. However, the geological age is very uncertain. In our opinion the most probable age is 2,800 Myr. The smallest time interval (200 Myr) is for the Lewisian gneisses. In other cases the time interval between Nd model age and granulite facies metamorphism is from 500 to 1,200 Myr.

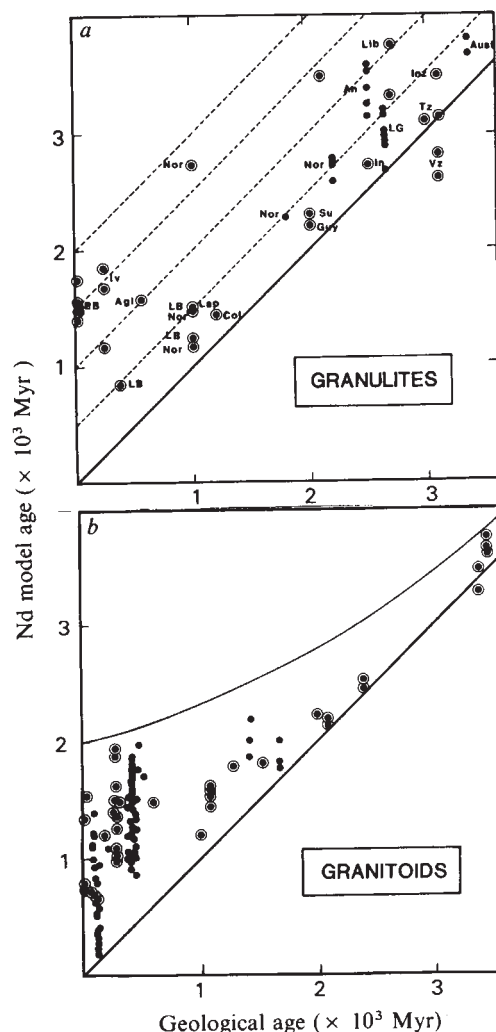
On the other hand, age determinations seem to indicate that granulite-facies metamorphism seems always to be linked to major orogeny. It does not reflect a continuous reequilibration process at depth stopped by the upwelling of a piece of lower crust to the surface during some event not related to any large orogenesis. If such reequilibration at depth followed by 'upwelling' occurs, this event is related to a major orogenesis.

Geological consequences

Following McCulloch and Wasserburg³¹ and O'Nions *et al.*⁵⁹, we interpret the Nd model age as the age of segregation from the mantle (EXCOD). The other ages, either the global geological ages or the better defined metamorphic ages, that we use for comparison with the Nd model ages, are clearly linked to orogenic processes and are sponsored to date INCOD. If this dual identification is correct we can draw the following conclusions: EXCOD has preceded INCOD by a large time interval for young granulites and for many of the older ones too. In these cases it seems that EXCOD and INCOD are two separate phenomena with INCOD operating on preexisting continental crust. This is only true if rocks have not been multiply metamorphosed in granulite facies.

For some Precambrian samples, ages of EXCOD and INCOD are similar. EXCOD and INCOD are still possibly two separate processes but would follow each other with a rather small time interval. The model proposed by Moorbath⁴³ for a single episode

Fig. 4 Nd model age versus geological age diagram. *a*, For the granulites, our data (●) are plotted together with other data (●) from refs 29, 30, 32–35. Underlined name samples correspond to well-known INCOD. *b*, For the granitoids, our data (●) are from 14, 20, 52, 74, the others (●) from refs 15–19. Inz: In'Ouzzal formation (Ahaggar)^{100–105} the primary age of these rocks is 3,300 to 3,100 Myr (ref. 41 and C.J.A. and D. Rousseau, in preparation) and the granulite metamorphism is 2,100 Myr¹⁰⁵ old. This age corresponds roughly to the age of a major orogeny in this area and to the age of granitic intrusions (Tassendjanet pluton). Lib: Liberian granulites, from Nimba Range and Grand Cape Mountains. The major tectonometamorphic event in this province is 2,800 Myr to 2,700 Myr old^{46,89} but 2,000 Myr ages corresponding to the Eburnean orogeny can be found. Granitoids in this area are also 2,600 to 2,700 Myr old^{46,89}. Vz: Imataca complex (Venezuela)^{45–47} the geological age of the basement is 2,800 to 2,700 Myr old⁴⁷ the major metamorphic event is 2,000 Myr old. Granitoids intrusions occurred at 2,700 Myr^{45,46} and 2.1 Myr. Guy: Guyana complex (Kanuku and Bakhuis Mountains)^{47,48,75,106}. Basement is 2,060 Myr for the Bakhuis Mountains¹⁰⁶ and 2,180 Myr old for the Kanuku formation⁷⁵. The major tectonometamorphic event and the related large granitic complexes is the 2,100–2,000 Myr transamazonian event⁷⁵. Ug: Western Nile complex (Uganda)^{107–109}. The granulites had been dated, not very accurately, at 2,600 Myr⁸⁸ and granitoid intrusion is 550 Myr old¹⁰⁹. Tz: Tanzania granulites from Msagali area. A poorly defined isochron gives an age of 2,690 Myr⁸⁸ but older ages may be possible. Granitoids from the north (Tanganikan shield) and south (Ubedian granites) are 1,800–2,000 Myr old¹⁰⁸. The whole system seems to have been affected by the Mozambican orogeny: 1.1–0.65 Myr ago¹⁰⁸. In: Granulites from Mysore and Madras State (India)⁹⁸. The granulites and probably the granitoids are 2,700 Myr old^{23,24,110}. The age of the granulite facies metamorphism is still debated: 2,000 Myr¹¹¹ or about 2,500 Myr¹¹². Col: Santa Martha Sierra (northern Colombia). The granulites are 1,270–1,370 Myr old^{76,113}, granitoids in this area are about 1.0 Myr old. Nor: Granulites from south Norway (Bamble, Rogaland). Rogaland: granulites are dated at 1,000–1,200 Myr¹¹⁴ and granulite metamorphism is supposed to be 1,000 Myr old¹¹⁵ but a possible metamorphic event may have occurred at about 1,400–1,500 Myr^{78,79,115}. Later intrusions took place at about 900–950 Myr^{116,117}. Bamble: ^{50,51,118} This region has suffered two major metamorphic events: 1,600 Myr (Svecofennian) and 1,000 Myr (Sveconorwegian) the latter being HP event⁴⁹. Granites are about 1,000 Myr^{50,51}. Lap: Valjok area (Lapland). Granulite metamorphism is supposed to be 1,000 Myr old (J. Touret, personal communication). LB: Bournac pipe, Massif Central, France (refs 40, 41, 63, 119 and J. Hamet and C.J.A., in preparation). An age of at least 1,000 Myr has been found for the first (granulitic) metamorphic event, followed by an hercynian event. Agl: Ansignan and Agly para- and ortho-derived granulites (Pyrenees, France)⁵⁴. A first metamorphic event occurred at 550 Myr. Later the granulite metamorphism is probably hercynian, related to the emplacement of the Pyrenean granitoids (350 Myr). BB: Beni Bousera granulites (Rif, Morocco) (refs 120, 121 and M. Polvé and C.J.A., in preparation). U-Pb, Sm-Nd, Rb-Sr dating show that the recycled material is very old and the granulite facies is young (20 Myr). Iv: Valle Strona-Ivrea zone (Italian Alps). This complete section of upper mantle, lower and intermediate crust is well known 122–127. The recycled material of the para-derived samples is 1,600 Myr–2,500 Myr old (ref. 128 and M. Polvé and C.J.A., in preparation). Two metamorphic events are known around 475 Myr⁹⁰ and 260 Myr (M. Polvé and C.J.A., in preparation). Granitic intrusions in the neighbouring zones are 270–275 Myr old⁹⁰.



of crustal differentiation may work in special cases but does not seem to be generally the case. A similar diagram can be constructed for granitoids for which the geological ages are well defined and correspond to the age of the intrusion^{14–17, 19,20} and compared with that for granulites (Fig. 4b). This comparison emphasizes two points: for young samples the two diagrams are very similar and thus recycling processes appear to be important in both cases. For old samples they are largely more scattered in the granulite cases than in the granitoid one.

A direct comparison between lower and upper crust is difficult at this stage, since upper crust contains large amounts of granitoid rocks but also a large proportion of gneisses and metamorphic terranes, for which the relative volumes are unknown. Granitoids seem to have a history that is easier to decipher and to systematize than that of granulite rocks, but we must remember that granitoids are produced by the melting of large volumes of rocks (including crustal rocks). Therefore such processes may homogenize relatively large volume of rocks giving a kind of statistical regional average.

Nd model ages are not sensitive to crustal events. Comparing Sr and Nd model ages, a Rb systematic depletion in lower crust should correspond to Sr model ages superior to Nd model ages.

Our data (Table 1) show that this case exists but the reverse (Sr model ages < Nd model ages) also exists, sometimes within the same area. This demonstrates the non-systematic depletion of Rb.

Conclusions

Granulite-facies rocks have Sm/Nd ratios identical to those of granitoids from the upper crust. These values are roughly constant for continental crust material (about 0.121 for ¹⁴⁷Sm/¹⁴⁴Nd). Thus internal differentiation of the continental crust apparently involves no fractionation of Sm and Nd. The Rb/Sr ratios are more variable and there is no unique signature characterized by systematic depletion in Rb. In some areas, rocks appear depleted in Rb while in others they are not. Such variability in the lower crust should be considered seriously. If on the average, the lower crust is more depleted in K, Rb, U and Th than the upper crust, as shown by heat flow studies⁶⁰ and/or budget calculations^{61,62}, the reason may be that the lower crust contains a larger proportion of basic rocks than the upper crust. If this hypothesis proves to be true, we shall have to change our estimates of the lower crust composition by taking

an average of acid and basic components. For the Sm/Nd ratios this is unlikely to change dramatically the estimates based on the present study, since basic intrusions, either tholeiites, alkaline or calcalkaline basalts, have a light REE enriched pattern resembling that of granitoids⁶³. On the opposite, the average Rb/Sr ratio of the lower crust will probably be considerably lowered. Future work on granulites should aim to estimate the respective proportions of basic and acid rocks in the lower crust.

The model-ages relationship clearly shows that recycling processes are important factors for building the lower continental crust. In most cases the lower crust is made up of preexisting igneous or sedimentary material. As shown by Touret⁵¹, granulitic rocks also contain a large proportion of metasediments. If these characteristics are true, and assuming that granulites are really the lower part of the continental crust, it is possible to propose a model for continental development: most of the new continental segment is developed on a basin,

located at the edge of the continent but with a continental basement. This basin is stressed, folded, intruded by mantle-derived melts, subjected to anatexis at several levels, and then transformed in a new continental segment. This segment has then the property that its upper part is made of new continental material intruded by anatectic granites which are partly reworked lower crust, partly locally derived, with its lower part made of older continental material. A small basin is a possible location for such an episode of continental growth. This model fits the constraints given by the petrogenesis of granitoids and granulites as determined by geology, petrology and isotope studies.

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Effect of chromosomal position on amplification of transfected genes in animal cells

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A single protein (CAD) contains the first three enzymatic activities of de novo uridine biosynthesis. The chromosomal location of CAD genes introduced into Chinese hamster ovary cells significantly affects the frequency and cytogenetic result of their amplification. The amplification frequency in one transformant is 100-fold that of the others; in another, amplification of donated genes inserted near a centromere results in chromosome instability and rearrangements.

GENE amplification in mammalian tissue culture cells occurs spontaneously, with high frequencies, and is a common mechanism of resistance of animal cells to diverse agents¹. Phenotypic reversion of mutations engendering temperature-sensitive² or kinetically altered³ proteins also occurs by gene amplification. Cytogenetically gene amplification results in expanded chromosomal regions which band abnormally by the trypsin-Giemsa method (often called homogeneously staining regions, or HSRs) or acentric extrachromosomal elements (called double minute chromosomes, or DMs) (see ref. 4 for a review). Since the amplified regions are 100–2,000 kilobases (kb)^{4–10}, far longer than the structural genes under selection, it is likely that chromosomal sequences or structures distant to the selected gene are involved in amplification. Since large amounts of DNA are co-amplified with the selected gene, 'sequence' amplification is often a more accurate term.

The abnormal chromosomal structures typical of gene amplification in drug-resistant tissue culture cells also occur in untreated spontaneous tumour cells^{11–15}. Cellular analogues of retroviral oncogenes are sometimes amplified in HSRs and DMs^{11,14,15}. Amplified proto-oncogenes are overexpressed in several tumour lines^{12,14,15} supporting the hypothesis that elevated expression of cellular oncogenes could be involved in tumorigenesis (see refs 16–18 for reviews).

The numerous examples of gene amplification emphasize that it can occur in many chromosomal regions. As in bacteria¹⁹, the chromosomal position of a mammalian gene can determine the amplification frequency and mechanism, and the size and stability of the resulting amplified regions. The various examples of eukaryotic sequence amplification differ significantly, reflecting the differences between cell lines and the selective agents used as well as the chromosomal positions of the genes. In order to assess directly the contribution of chromosomal position in sequence amplification, we studied the amplification of a cloned CAD gene inserted by gene transfer into different locations in the chromosomes of CAD-deficient Chinese hamster ovary cells (CHO) cells. [CAD is a multifunctional protein which contains the first three enzymatic activities of *de novo* uridine biosynthesis: carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase.]

High-frequency amplification

Purified DNA sequences introduced into mammalian cells integrate into chromosomes in an apparently random fashion^{20–22}. If chromosomal position affects gene amplification, it should be possible to use gene transfer to screen for chromosomal sites which engender unusual forms of amplification. Two

parameters which could be altered by changing gene position are the frequency and the cytogenetic consequences, for example, rearrangements associated with amplification, of its amplification.

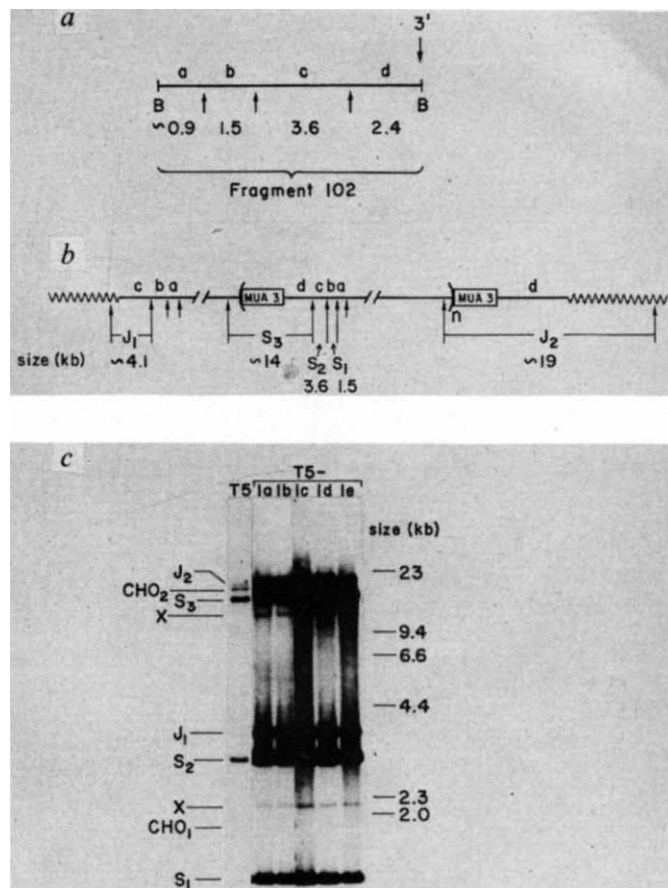
To establish whether either of these parameters is affected by changing gene position, we introduced cloned genomic copies of the Syrian hamster CAD genes²⁰ into the chromosomes of CAD-deficient (Urd-A)²³ mutants of CHO cells using the calcium phosphate precipitation technique in the absence of carrier DNA^{24,25}. Transformants expressing the donated Syrian hamster CAD genes can be identified by their ability to grow in the absence of uridine²⁰. Four transformants were isolated which contain approximately three to six functional donated CAD genes. The CAD activity in each transformant was approximately the same as that of the wild-type CHO K1 cells (see Table 1). This agrees with our previous finding that the Syrian hamster CAD gene is expressed at approximately one-quarter the level of the Chinese hamster CAD gene²⁰. Each transformant maintained the donated genes without restriction pattern changes after passage for 90 generations without selection (data not shown).

Due to their similar levels of CAD expression, transformants and wild-type CHO K1 cells are sensitive to the same concentrations of PALA (*N*-phosphonacetyl-L-aspartate, see LD₅₀ in Table 1), a specific inhibitor of the aspartate transcarbamylase activity of CAD²⁶. Since PALA resistance has been found only when the CAD gene is amplified^{27,28}, we used the frequency of PALA resistance as an indicator of CAD gene amplification frequency. Table 1 shows the number of PALA-resistant (PALA^r) colonies that arose in transformants T1, T5, T6 and T7 at the three PALA concentrations tested. Transformant T5 differs from wild-type Syrian hamster²⁸, from CHO K1 cells and from the other transformants: in T5 the frequency of PALA resistance is at least 100 times greater and T5 resists higher concentrations of PALA in a single step even though the other transformants have equivalent or higher aspartate transcarbamylase specific activities. All of the PALA^r T5, T6 and T7 clones tested maintain their initial levels of PALA resistance when grown for 60 generations in the absence of the drug, indicating that PALA resistance is relatively stable in these lines. The amplified CAD genes in one PALA^r T1 mutant appeared to be much less stable (see below).

To determine whether high frequency PALA resistance in transformant T5 results from amplification of donated CAD sequences, DNA isolated from the parental transformant and from five independently isolated PALA^r mutants of T5 was digested with endonuclease SstI and then analysed by blot hybridization^{29,30} using the CAD-specific probe P102³¹ (Fig. 1). The bands representing the endogenous CHO CAD genes (labelled CHO₁ and CHO₂) are easily resolved from the three Syrian hamster CAD gene bands (labelled S₁, S₂, S₃). It is clear that the donated sequences are amplified in each of the PALA^r

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Fig. 1 Analysis of the donated CAD genes in transformant T5 and in PALA^r mutants. **a**, Map of CAD gene fragment 102. 102 is an 8.6-kb *Bam*HI (B) fragment derived from the 3'-end of the 25–30-kb Syrian hamster CAD gene and contains only unique sequences³¹. The map of fragment 102 published previously had an inversion of fragments b and c²⁰. ↑ indicates *Sst*I cleavage sites. **b**, Simplified map of the inserted sequences in transformant T5. The donated array consists of four to six CAD genes in a head-to-tail tandem array (one indication of this arrangement is the presence of fragment S₃ which has been shown by hybridization analysis to contain sequences at the 5' end of one CAD gene, vector sequences, and the 3' end of another CAD gene). Only the relevant *Sst*I sites (↑) are shown. Not to scale. **c**, Hybridization of *Sst*I-digested DNA isolated from T5 and PALA^r T5 mutants with fragment 102. The lanes indicate the clones used as the source of the genomic DNA. T5 is the parental transformant, and T5-1a to T5-1e are independent PALA^r mutants isolated from T5 after selection in 250 μM PALA. Each mutant was expanded from a single, well-isolated colony for DNA isolation. Mutant T5-1d is the same as T5-S1-3 shown in Figs 2 and 3. Exposures, which show the endogenous CHO CAD gene fragment CHO₂ more clearly, demonstrate that all lanes contain approximately the same quantity of DNA. CHO₁ and CHO₂ indicate the Chinese hamster CAD gene fragments, S₁, S₂, S₃ are the donated Syrian hamster CAD gene fragments, J₁ and J₂ are the joints between the donated and CHO DNA, and X is a fragment produced by inefficient *Sst*I cleavage which is also seen after cleavage of CAD cosmid DNA. **Methods:** Cellular DNAs (10 μg) were digested with a 5–10-fold excess of *Sst*I, fractionated by electrophoresis through a 0.7% agarose gel, and analysed by the Southern blot hybridization technique^{29,30}. The DNA was dehydrated *in situ* in 0.25 M HCl for 10 min (instead of 20–30 min as described previously³⁰). The filter was hybridized with 3 × 10⁶ c.p.m. per ml of nick-translated⁴⁷ p102 (see Fig. 1a) in the presence of 10% dextran sulphate for 16 h and washed as described previously³⁰.



T5 mutants. Three other conclusions derive from this experiment. First, even though each T5 mutant was isolated after a single step of selection, there is an approximately 25–50-fold increase in copy number (determined by densitometry of autoradiographs exposed in the linear range of the film). This compares with a 5–10-fold increase in PALA^r mutants of other transformants (for example, see results below for transformant T1). Second, the molecular structure of the amplified donated sequences is identical in all independently isolated T5 mutant. Third, amplification of the donated CAD genes in T5 does not result in their rearrangement. The bands labelled 'X' were observed after restriction digestion of purified cosmid DNA, and probably represent low efficiency cleavage sites for endonuclease *Sst*I. Fragments 'J₁' and 'J₂' are derived from the junctions between the donated and flanking CHO DNA as described below.

Amplification mediated by flanking sequences

DNA blotting experiments shown above for transformant T5 and below for transformant T1 (data not shown for the other transformants) demonstrate that donated CAD genes can be amplified in each transformant. All of the transformants were derived from the same recipient cell line (Urd-A), and all were transformed with the same CAD cosmid. Thus, the capacity for high frequency amplification in T5 is not related to the DNA used or to some general characteristic of the Urd-A cells following transfection. Since the frequency of dihydrofolate reductase (DHFR) gene amplification is essentially the same in CHO K1, Urd-A and T5 (data not shown), T5 is not a genetic variant capable of amplifying all sequences at a high frequency. Therefore, the high frequency amplification in T5 relates either to the organization of the donated sequences or to an effect of the position in which they are inserted.

The restriction analysis shown in Fig. 1c indicates that T5 contains four to six donated genes (for example, compare the

intensities of S₁–S₃ with those of CHO₁ and CHO₂). The *in situ* hybridization analysis in Fig. 2 shows that the donated genes in T5 are contained in a single chromosomal location and that amplification occurs near to or at this site without obvious chromosome rearrangements. Hybridization analysis with different probes has shown that fragment S₃ (Figs 1c, 3) contains sequences at the 5' end of one CAD gene, the adjacent vector, and the 3' end of the next CAD gene, indicating that the donated genes are in a head-to-tail array (see Fig. 1b). From previous studies, we infer that the tandem array arose by homologous recombination either before or after chromosomal integration^{22,32}. Since tandem arrays mediate amplification by unequal sister chromatid exchanges in prokaryotes¹⁹ and yeast³³, we tested whether such exchanges occurring within the tandem array in T5 cause the observed high frequency amplification.

Reciprocal unequal exchanges within the tandem array of CAD genes should generate two chromatids with unequal numbers of CAD genes. After mitosis and cell division, only the daughter cell receiving the chromosome with the greater number of CAD genes will survive the PALA selection. Thus, the abundance of joint sequences between donated and CHO DNA should not increase following amplification by unequal exchange since half of the joint sequences are lost in the cell which does not survive the selection. To test this prediction, we identified the joints between the donated and CHO DNA and measured their abundance after amplification in five independently isolated mutants.

Since there is a single insertion site in T5, the joints between the CHO and donated DNA should be contained in two restriction fragments which do not correspond to the known CAD gene restriction map. These fragments should have unique sequence abundance. Fragments J₁ and J₂ (Fig. 1c), which hybridize with fragment 102, are the only two *Sst*I restriction fragments which fulfill these criteria. Furthermore, since probes for other regions of the CAD gene do not reveal other novel fragments, the sequence disruption caused by gene insertion is

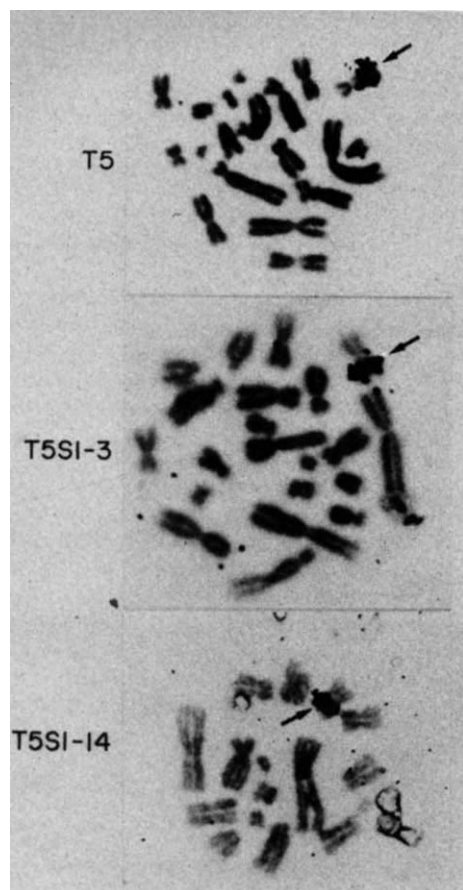


Fig. 2 Localization of the donated CAD genes in T5 and PALA^r T5 mutants by *in situ* hybridization. Cell lines used for the metaphase spreads are indicated in each panel. Hybridization to the chromosomes indicated by the arrows represents the only significant hybridization observed in 10 metaphase spreads from each cell line. The dense packing of grains in these exposures, the unequal hybridization to chromosomes prepared from the different cell lines at different times, and the uneven hybridization seen across the slides prevents these data being used to determine the relative number of CAD sequences in each cell line.

Methods: Methods for spread preparation and *in situ* hybridization have been described in detail previously and were modified only by use of ³H-labelled rather than ¹²⁵I-labelled p102⁵. The probe was nick-translated p102. Exposures were for 2 weeks at -70 °C.

localized to fragment 102 (see Fig. 1b). When subfragments 102C and 102D (Fig. 1a) were hybridized to the blot shown in Fig. 1c, subfragment 102C hybridized preferentially with J₁ and S₂, and subfragment 102D and vector sequences hybridized with both J₂ and S₃ (Fig. 3). These data agree with the map in Fig. 1b. The results show that J₁ and J₂ are the joints between the donated and CHO DNA, and demonstrate that the joint fragments are amplified in the PALA^r T5 mutants to the same extent as the CAD sequences which they flank (see Figs 1c, 3). Thus, it is unlikely that amplification of the donated genes in T5 occurs by reciprocal unequal exchanges within the donated sequences. The data indicate that the amplified unit extends beyond the CHO-donated DNA junctions, implying that high frequency amplification in T5 is related to the position of the inserted sequences in the genome.

Chromosome rearrangements

The genetic, molecular and cytogenetic aspects of amplification of the donated genes in transformant T1 contrast with those

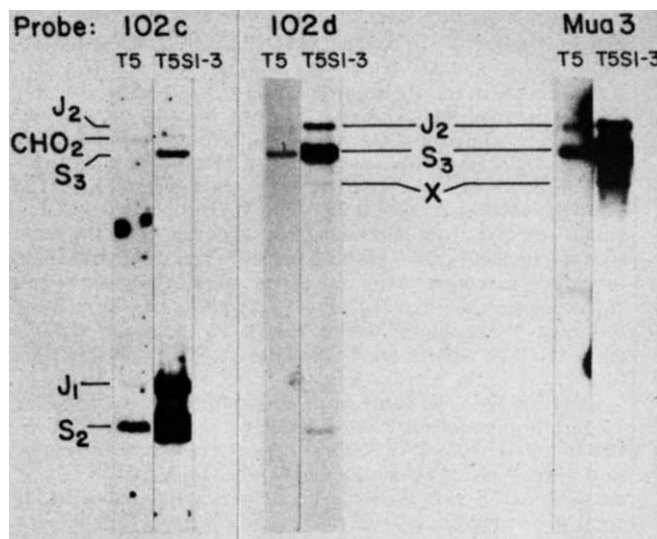


Fig. 3 Analysis of joint fragment amplification in transformant T5 and PALA^r mutant T5-SI-3. The blot used in Fig. 1 was washed to elute bound counts (see below) and then hybridized with the indicated probes. For simplicity, we show the pattern of hybridization to DNA isolated from only one PALA^r T5 mutant since all the hybridization patterns from the five mutants analysed were identical (for example, Fig. 1c).

Methods: The blot shown in Fig. 1 was washed with boiling 0.1 × SSPE, 0.1% SDS briefly to remove the bound counts. Subsequent hybridization reactions contained 3–5 × 10⁶ c.p.m. per ml indicated probes, and all reactions were performed as described in the legend to Fig. 1. Film exposures: 84 h for 102c, 72 h for 102d; 120 h for probe MUA3. Reduced hybridization signals were seen with probe 102d because it was 2 weeks old at the time it was used.

observed in transformant T5. The frequency of amplification (10⁻⁵–10⁻⁶), maximal level of amplification (5–10-fold) and resistance to PALA attained in a single step are similar in T1 and wild-type CHO K1 cells (see Table 1). In contrast to transformant T5 (Fig. 2), CAD gene insertion in T1 is accompanied by numerous CAD gene restriction fragment rearrangements (Fig. 4). Since T1 contains an *Sst*I restriction fragment which co-migrates with and has the same hybridization characteristics as fragment S₃ of transformant T5, T1 must also have a core of CAD genes present in a head-to-tail tandem array. The numerous other *Sst*I fragments which hybridize with fragment 102 indicate this tandem array is surrounded by rearranged CAD sequences (two fragments are approximately the same size as CHO₁ and CHO₂ and obscure them). All of these sequences amplify to the same degree without further rearrangements (see Fig. 4, lane T1-1), indicating the amplified unit extends beyond the boundaries of the inserted DNA.

Figure 5 shows the location of the inserted sequences in T1 and the cytogenetic consequences of their amplification. A single insertion site coinciding with the centromeric constriction of a large telocentric chromosome was seen in the 12 metaphase spreads examined. Amplified sequences in independently isolated PALA^r mutants of T1 were found in different chromosomal locations. In mutant T1-1, an entire arm of one medium-sized metacentric chromosome contains most if not all of the amplified sequences. A second chromosome which is very similar to the parental chromosome with regard to the number and location of donated sequences was also found in 14 out of 15 spreads of T1-1. By contrast, the amplified CAD sequences in T1-2 often reside in one or more small chromosomes or chromosome fragments (see Fig. 5, panels T1-2). Preliminary studies of T1-2 show that after passage in the absence of PALA for 40 generations gene copy number decreases by at least half and PALA resistance is diminished. In five out of eight spreads

Fig. 4 *Sst*I fragments in T1 and PALA^r mutant T1-1 which hybridize with CAD fragment 102. The lanes indicate the clones used as the source of the DNA. T1 is the parental line and T1-1 is a mutant selected to resist 250 μ M PALA. The positions of the normal Syrian hamster fragments are indicated by S₁, S₂ and S₃ (see Fig. 1b). The other fragments come from rearranged Syrian hamster CAD fragments. The restriction digestion, blotting and hybridization conditions are described in the legend to Fig. 1. The ethidium bromide staining pattern showed about twice as much T1-1 as T1 DNA. The numerous rearranged Syrian hamster bands obscure hybridization to the CHO CAD gene fragments. Overexposed autoradiograms showed that all CAD sequences amplified in T1-1 are found in T1, and all amplify to the same extent.

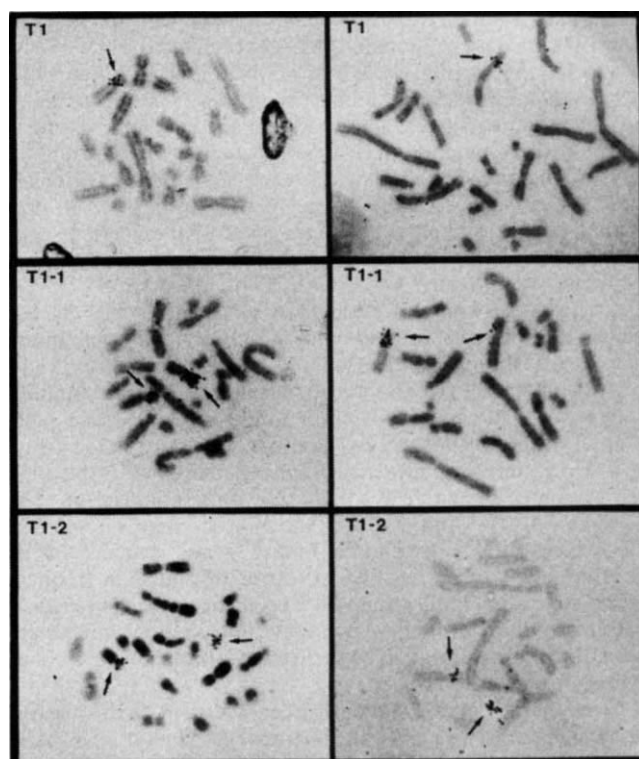
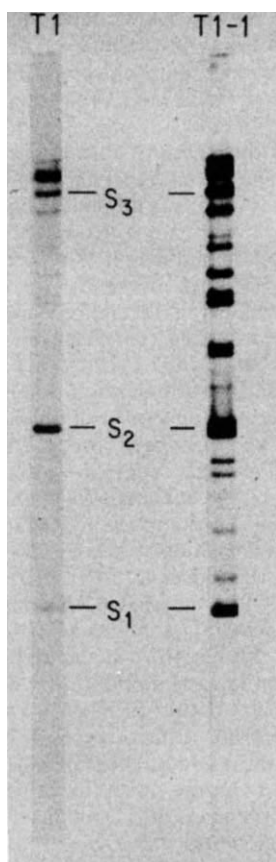


Fig. 5 *In situ* hybridization analysis of T1, T1-1 and T1-2. Representative metaphase chromosome spreads from the indicated cell lines were hybridized with ³H-labelled p102 as described in the legend to Fig. 2.

of T1-2 the donated genes occurred in a chromosome which resembles that found in the parental transformant (T1) and two out of eight metaphase spreads revealed amplified sequences at chromosome telomeres.

Discussion

We used *in vitro* gene transfer to introduce cloned CAD genes into new chromosomal locations in CAD-deficient CHO cells to determine whether position affects the frequency or cytogenetic result of CAD gene amplification. We found that both can be affected significantly by gene position. For example, the donated genes in transformant T5 amplify approximately 100 times as frequently as those in three other transformants

or the endogenous CAD genes in several wild-type cell lines. Furthermore, the donated genes in T5 are amplified 25–50-fold after the first selective step in contrast to the 5–10-fold increases seen in the other transformants or several wild-type lines^{27,28}.

The transfected CAD genes in T5 are in a head-to-tail tandem array 200–300 kb long. Within the array, each CAD gene contains *Alu*-like elements repeated in many introns³⁴ and the vector sequences are repeated every 40 kb. Such a structure should provide many opportunities for amplification mediated by homologous unequal exchanges. However, we have found no evidence that high frequency amplification in T5 results from reciprocal unequal exchanges within the tandem array. The data agree better with an amplified region which includes the donated sequences and extends into the flanking chromosomal DNA. The amplified region in the three other transformants examined

Table 1 Characterization of transformants T1, T5, T6 and T7

Cell line or transformant	CAD specific activity (μ mol per mg protein per 30 min)	LD ₅₀ (μ M)	Frequency of resistance (colonies/ 3×10^5 cells)		
			100 μ M PALA	After selection in 250 μ M PALA	1 mM PALA
CHO-k1	0.27	20–30	ND	3	0
T1	0.17	5–10	0	1	0
T5	0.19	5–10	130	152	75
T6	0.33	20–30	1	ND	0
T7	ND	20–30	1	ND	0

CAD-specific activity was determined by measuring the ATCase activity as described previously²⁰. The LD₅₀ refers to the PALA concentration (μ M) required to reduce the plating efficiency of 10^3 cells per 35-mm dish by 50%. The frequency of PALA resistance was determined by seeding 3×10^5 cells per 10-cm dish, allowing attachment to occur overnight, and then challenging the cells in the indicated concentrations of PALA. The number of colonies which developed after 3–4 weeks of selection were then determined by counting the plates after fixing with methanol and staining with Giemsa. ND, not determined.

also extends into CHO DNA. The donated unit in these lines consists of a core of tandem CAD genes flanked by rearranged CAD sequences (Fig. 4). Thus, neither intact nor rearranged CAD sequences can mediate their own amplification by reciprocal unequal exchanges which occur solely within the donated array: flanking CHO DNA participates in the amplification of the transfected genes in each transformant. Hence, if unequal exchanges can cause sequence amplification in mammalian cells, it is likely that factors other than extended regions of sequence homology are required to initiate or mediate the process.

One model for amplification in T5 which agrees with the large single step increase in gene copy number is that unscheduled rounds of synthesis of the donated sequences are followed by recombinational events to generate a stable amplified region³⁵⁻³⁷. Unscheduled DNA synthesis mediates chorion gene amplification in *Drosophila*³⁸ and has been postulated to lead to DHFR gene amplification in mammalian cells³⁹, the *in situ* replication of simian virus 40 and polyoma sequences in transformed cells^{36,40} and the amplification of adenine phosphoribosyl transferase-thymidine kinase genes transfected into mouse cells³⁷. It is tempting to speculate that high frequency and high level amplification in T5 occur because gene insertion disrupts a domain or sequence that controls the replication of an adjacent region, thereby allowing frequent unscheduled synthesis of the donated array.

Gene amplification frequently occurs at sites of chromosome rearrangements^{5,41,42}. While obvious cytogenetic changes did not occur in PALA^r T5 mutants, significant chromosomal rearrangements did occur during amplification of the donated genes in T1. Interestingly, the CAD genes were inserted near to a centromere in T1 but in the middle of a chromosome arm in T5. PALA^r T1 mutants retain the chromosome with the donated

genes and contain the amplified copies of these genes in another chromosome (in T1-1) or in extrachromosomal elements or small chromosomes (in T1-2). Similar observations to those made in T1 have been reported for the amplification of sequences in IMR-32 neuroblastoma cells⁴³. The apparent retention of the parental chromosome and the appearance of amplified sequences in other chromosomes in PALA^r T1 mutants suggests that sequence amplification near or at a centromere is lethal in cells with only one copy of this chromosome. Perhaps an event such as nondisjunction is first required to generate a cell with two copies of the chromosome containing the inserted CAD genes. Amplification in one of these chromosomes could then generate a dicentric chromosome which undergoes a breakage-fusion-bridge cycle⁴⁴ to yield chromosome fragments which attach to telomeres of other chromosomes as in T1-1, or remain free and engender an unstable phenotype as in T1-2. A similar model has been proposed recently to explain the frequent telomeric location of amplified DHFR minigenes in CHO cells⁴². On the other hand, the maintenance of sequences in one chromosomal site and their appearance in a second site characterizes transposition⁴⁵. If amplification is associated with transposition of sequences in T1, the transposed sequence must be at least 300 kb, the size of the inserted sequences! Moreover, since *Drosophila* contains elements capable of transposing sequences of this length⁴⁶, the involvement of large-scale DNA transposition in gene amplification deserves further consideration.

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Close similarity of epidermal growth factor receptor and *v-erb-B* oncogene protein sequences

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Each of six peptides derived from the human epidermal growth factor (EGF) receptor very closely matches a part of the deduced sequence of the v-erb-B transforming protein of avian erythroblastosis virus (AEV). In all, the peptides contain 83 amino acid residues, 74 of which are shared with v-erb-B. The AEV progenitor may have acquired the cellular gene sequences of a truncated EGF receptor (or closely related protein) lacking the external EGF-binding domain but retaining the transmembrane domain and a domain involved in stimulating cell proliferation. Transformation of cells by AEV may result, in part, from the inappropriate acquisition of a truncated EGF receptor from the c-erb-B gene.

REGULATION of the proliferation of cells in culture can be influenced by a number of mitogens including a series of polypeptide growth factors which, acting alone or synergistically with other mitogens, can induce DNA synthesis and proliferation of specific target cells (for recent reviews see ref. 1). Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are probably the best characterized growth factors: the precise function of these polypeptides *in vivo* is, however, unclear. EGF may have a role in cell proliferation and differentiation since it will induce early eyelid opening and incisor development in new born mice²; PDGF on the other hand, which is released from platelets during blood clot formation at wound sites, may have a role in repair processes³. These and other growth factors *in vitro* can trigger a variety of morphological and biochemical changes that resemble those characteristic of transformed cells, and have also been implicated in the abnormal regulation of proliferation shown by transformed and tumour-derived cell lines (reviewed in refs 4 and 5). Thus it has been suggested that transformed cells may both synthesise and respond to growth factors and consequently proliferate independently through 'autocrine' secretion⁶. Direct support for such an autocrine role for aberrantly expressed growth factors in the control of abnormal cell proliferation came recently from the discovery that the putative transforming protein (p28^{sis}) of simian sarcoma virus (SSV) is structurally related to the growth factor PDGF⁷⁻⁹ and can also function like PDGF as a growth factor for cells in culture¹⁰. Other growth factors produced by transformed cells such as insulin-like growth factor (IGF)^{11,12}, fibroblast-derived growth factor^{13,14} and the transforming growth factors (TGFs)¹⁵⁻²⁰, may also act as autocrine regulators of proliferation. Besides the specificity mediated by regulation of the production of growth factors, cellular specificity could also be controlled at several other levels—the most obvious being by binding of ligand to specific receptors present only on target cells. In addition the binding of one growth factor to its specific receptor can also alter the affinity of another growth factor for its receptor (for example, PDGF and the EGF receptor^{21,22}). Conversely two growth factors may, as appears to be the case with α TGFs and EGF, bind to the same receptor^{23,24}.

It is clear that binding of different growth factors to their specific receptors can induce a cascade of biochemical events including rapid changes in ion movements and intracellular pH, stimulation of tyrosine specific protein kinases and several other changes which can culminate in DNA synthesis and proliferation

of certain target cells^{1,4-6}. It seems likely that at least in the case of the EGF receptor the primary function of EGF may be to induce cross-linking or conformational changes of receptors, and that following such an activation step, all the 'information' necessary for triggering a proliferative response may reside in the receptor itself (see ref. 1). One known function intrinsic to the EGF receptor is its ability to phosphorylate tyrosine residues²⁵⁻²⁸, a property shared with five of the putative transforming proteins of the family of retroviruses whose oncogenes are structurally related to *src* but not by two others, the proteins encoded by *mos* and *erb-B*²⁹. At present this tyrosine kinase activity provides the only functional activity associated with the oncogenes of this subset of retroviruses.

Here we report amino acid sequence analysis of six distinct peptides from human EGF receptors isolated by monoclonal immunoaffinity purification from A431 cells and placenta, and show that 74 out of 83 of the residues sequenced are identical to those of the transforming protein encoded by the *v-erb-B* oncogene of avian erythroblastosis virus (AEV)³⁰. The amino acid sequences of several other peptides purified from the EGF receptor could not be aligned with sequences of the *v-erb-B* protein suggesting that AEV has acquired cellular sequences encoding only a portion of the avian EGF receptor. Several lines of evidence suggest that the *v-erb-B* oncogene encodes only the transmembrane region of the EGF receptor and the domain associated with the tyrosine kinase activity. These results suggest a hypothesis that the *src* related subset of oncogenes, which includes *v-erb-B*, may be derived from cellular sequences which encode growth factor receptors and produce transformation through expression of uncontrolled receptor functions.

Purification of EGF receptor

EGF receptors can be detected in a variety of cells either by measurement of EGF binding (reviewed in ref. 31), by cross-linking of labelled EGF to its receptor (reviewed in ref. 32) or by using monoclonal antibodies³³⁻³⁸. In this study the receptor has been purified from two sources: the human epidermoid carcinoma cell line A431, which expresses about 50 times more receptors than the majority of other cells^{39,40}, and human placenta⁴¹ which is a readily available normal tissue. The recent isolation of monoclonal antibodies which recognize the human EGF receptor^{34,38} has made it possible to use immunoaffinity chromatography for receptor purification. Here we compare by peptide mapping EGF receptor protein purified by either immunoaffinity or EGF affinity chromatography²⁶ and also compare the structures of the A431 and placental receptors.

A radioimmunoassay (RIA) which uses a monoclonal antibody (R1) has been used to quantitate various preparative

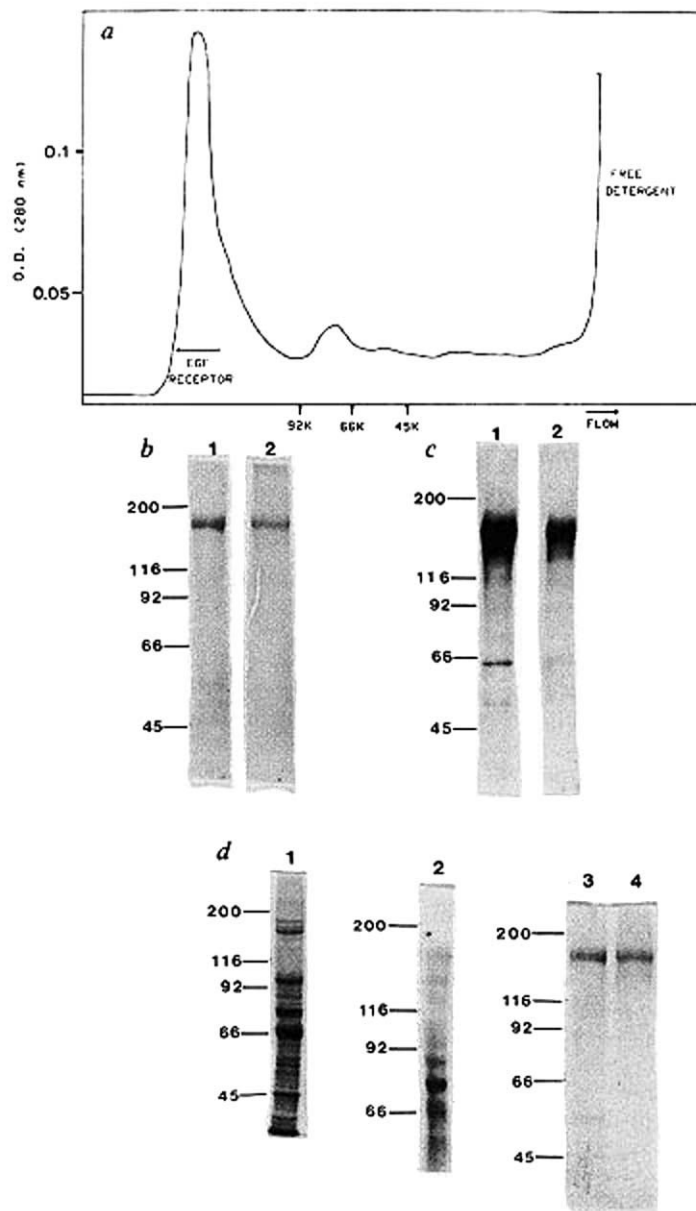
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Fig. 1 Immunopurification of EGF receptor from A431 cells and human placenta. **a**, Purification by gel permeation. Reduced and alkylated receptor was purified on a TSK4000 column (0.7 × 60 cm, LKB) using 0.1 M potassium dihydrogen phosphate buffer pH 4.5 containing 6 M guanidine HCl at a flow rate of 0.5 ml min⁻¹ (ref. 43). The absorbance of the eluate was monitored at 280 nm (molecular weights of protein standards are indicated) and 0.25 ml fractions were collected. Fractions containing EGF receptor were dialysed against 10 mM ammonium bicarbonate. Panels **b–d** show 7% polyacrylamide SDS gels⁷² used to monitor purification (MW × 10⁻³ of protein standards are indicated). **b**, R1 purified A431 EGF receptor: track 1, pH 3 eluate from R1 immunoaffinity matrix; track 2, eluate from the TSK4000 column. **c**, 29-1 purified A431 EGF receptor: track 1, pH 3 eluate from 29-1 immunoaffinity matrix; track 2, eluate from SDS preparative gel electrophoresis. **d**, Lectin and R1 purified placental EGF receptor: track 1, placental vesicles; track 2, eluate from lectin affinity matrix; track 3, eluate from R1 immunoaffinity matrix; track 4, eluate from the TSK4000 column.

Methods: Approximately 2 × 10⁹ A431 cells were washed in calcium and magnesium free phosphate-buffered saline (PBS) and solubilized in 400 ml lysis buffer (50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM EGTA, 0.1% bovine serum albumin, 1% NP40, 25 mM benzimidazole, 0.2 mM PMSF, 10 µg ml⁻¹ leupeptin). After filtration through muslin the lysate was adjusted to pH 8.5 and centrifuged at 100,000g_{max} for 30 min. The supernatant was incubated for 2 h at 4 °C with immunoaffinity matrix, which consisted of 15 mg of monoclonal antibody R1³⁴ coupled to 15 ml of Affi-Gel 10 (BioRad). Unbound lysate was removed by suction through a 0.4 micron filter. The matrix was then washed by gentle agitation and filtration with 500 ml PBS, containing 0.65 M NaCl and 0.1% NP40, followed by 500 ml PBS, containing 0.1% NP40. The EGF receptor was eluted by gentle agitation and filtration of the matrix with 2 × 10 ml aliquots of 50 mM sodium citrate pH 3, containing 0.05% NP40 for 10 min each. Eluates were adjusted to pH 7. The yield of receptor was approximately 250 µg, measured by Bradford technique⁷⁰ or by amino acid analysis after gel permeation HPLC (see below). Alternatively EGF receptor was purified from A431 cells using monoclonal antibody 29-1³⁸ coupled to CNBr-activated Sepharose (Pharmacia) at 5–10 mg ml⁻¹. The purification procedure used was similar to that described for the R1 immunoaffinity matrix except that the EGF receptor was phosphorylated whilst bound to the matrix with 50 µCi [γ-³²P]ATP (3,000 Ci mmol⁻¹, Amersham International) in the presence of 3 mM MnCl₂. Eluted receptor was further purified by preparative SDS gel electrophoresis, followed by dialysis against 10% methanol at 4 °C. For purification of placental EGF receptor, vesicles were made from syncytiotrophoblast microvilli by a modification of the method of Smith *et al.*⁷¹, using 2 mM EGTA in all buffers. Vesicles were solubilized by addition of an equal volume of 100 mM HEPES pH 7.4, 0.15 M NaCl and 5% Triton X-100. After centrifuging at 100,000g_{max} for 30 min the supernatant was incubated for 1 h at room temperature with 200 mg wheat germ agglutinin coupled to 10 g of Affi-Gel (BioRad). The lectin matrix was washed by filtration through a 0.4 µm filter with 100 ml of PBS, containing 0.1% Triton X-100. Bound protein was eluted by agitation and filtration with 2 × 15 ml aliquots of 0.25 M *N*-acetylglucosamine in 10 mM HEPES pH 7.4, 0.1% Triton X-100 for 15 min each. The eluate was incubated with R1 immunoaffinity matrix (15 mg antibody per placenta) for 2 h at 20 °C, followed by extensive washing and elution as described above for receptor purification from A431 cells. The yield of EGF receptor per placenta was 25 µg, measured by Bradford technique⁷⁰ and amino acid analysis after gel permeation HPLC (see below). Solutions containing EGF receptor were lyophilized and resuspended in 0.5 M Tris-HCl pH 8.5, 6 M guanidine hydrochloride (Schwarz-Mann) at 0.5–1 mg ml⁻¹. After incubation at 37 °C for 16 h with 10 mM dithiothreitol, cysteine residues were alkylated with ¹⁴C-iodoacetamide (40–60 mCi mmol⁻¹, Amersham International) as described previously⁴⁴.

techniques⁴². Receptors from A431 cells and placenta were both found to be unstable in detergent-solubilized whole cell or tissue lysates, perhaps as a result of the release of proteases from the cellular lysosomal compartment. This problem was overcome for placenta by the preparation of syncytiotrophoblast microvilli plasma membranes and as a result a 50-fold purification with a 30% yield of receptor was achieved. Unfortunately, with A431 cells the yield of receptor in plasma membrane preparations was impractically low. However, quantitative studies with the receptor RIA showed that rapid adjustment of the lysate pH to 8.5 followed by fast immunoaffinity chromatography of whole cell lysates minimised the effects of the proteases.

Placental membranes were solubilized and glycoproteins separated by wheat germ agglutinin (WGA) affinity chromatography to achieve a partial purification. EGF receptor was then purified from the placental glycoprotein fraction or from A431



cell lysates by immunoaffinity chromatography on either monoclonal antibodies R1³⁴ or 29-1³⁸ immobilised on agarose or Sepharose respectively. Nonspecifically bound protein was removed by washing the columns with a high salt buffer and the receptor was eluted at pH 3. The receptors were further purified either on preparative SDS-polyacrylamide gels or by gel permeation HPLC in guanidine solutions⁴³. Details of the methods used and the yields of purified receptors are given in the legend to Fig. 1. Since the EGF binding and protein kinase activity were partially destroyed during purification, receptor was also purified by EGF affinity chromatography²⁶. Comparative HPLC tryptic peptide maps were then carried out to establish the purity and structural similarity of receptor prepared by immunoaffinity chromatography from A431 cells and placental tissue. The peptide maps of the receptors (Fig. 2) showed that the elution profiles of the receptor tryptic peptides were very

similar whether receptor was purified by EGF affinity or by immunoaffinity chromatography; from A431 cells or from placental tissue.

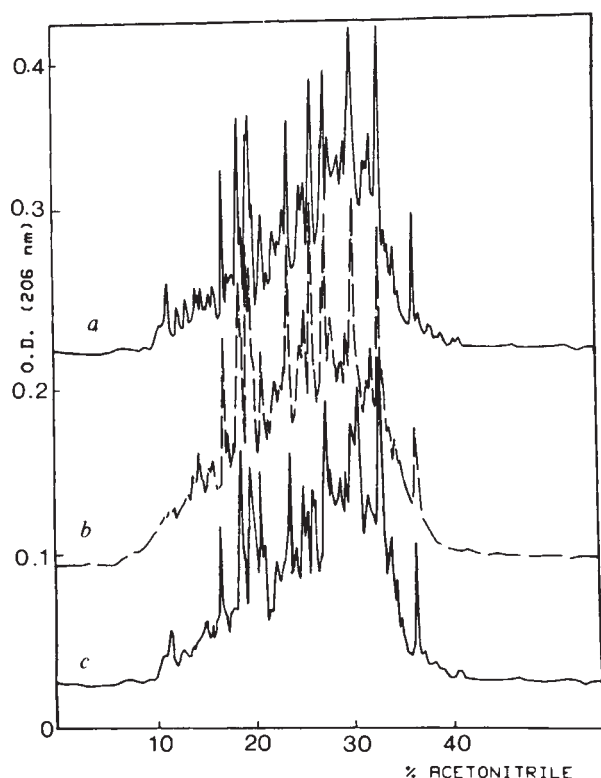


Fig. 2 Reverse phase HPLC analysis of tryptic peptides from EGF receptor purified by three different affinity methods. *a*, Immunoaffinity purified A431 receptor. EGF receptor was purified from A431 cells using the monoclonal antibody R1 and gel permeation chromatography in guanidine solutions as described in Fig. 1. Pooled fractions containing the 175,000 MW EGF receptor were dialysed against 10 mM ammonium bicarbonate, lyophilized and resuspended in 500 μ l of 100 mM ammonium bicarbonate, 10 mM CaCl_2 . TPCK treated trypsin (Sigma) was then added (100:1, receptor:trypsin, w/w). This mixture was incubated at 37 °C for 12 h, a further identical aliquot of trypsin added and the incubation continued for another 12 h. Peptides were then loaded directly onto a Synchropak RPP C18 reverse phase HPLC column (Synchrom, Linden, Indiana, 4.6 $\times$ 75 mm) equilibrated in 0.1% trifluoroacetic acid (TFA, Rathburn, Scotland) and a gradient from zero to 45% acetonitrile was run over 45 min at 1 ml min⁻¹ and 1 ml fractions collected⁴⁶. A Waters HPLC system including two M6000 A pumps, a U6K manual injector and a 660 solvent programmer with 2 LKB 2138 Uvicord S absorbance detectors with filters at 206 nm and 280 nm was used⁴⁵. The figure shows the optical density of the eluate at 206 nm plotted against acetonitrile concentration. *b*, EGF affinity purified receptor. EGF receptor was purified from A431 cells using wheat germ agglutinin affinity chromatography followed by EGF affinity chromatography²⁶. A431 cells were lysed as described in the legend to Fig. 1. Purification of this lysate on the WGA affinity column was identical to that described for the placental preparation (Fig. 1). The eluate from the WGA affinity column was mixed with 5 ml of Affi-Gel 10 (BioRad) having 1 mg of bound EGF. The mixture was tumbled for 4 h at room temperature before washing the immobilized EGF receptor with 100 ml of PBS, 0.1% Triton X-100. Receptor was then eluted with 10 mM ethanolamine, pH 9.7, 0.1% Triton X-100. This eluate was further purified on a TSK4000 gel permeation column as described in Fig. 1. Fractions containing EGF receptor were pooled, dialysed, lyophilized and trypsinized as described for *a* above. The resulting tryptic peptides were separated by reverse phase HPLC under identical conditions to those described above. *c*, Immunoaffinity purified placental receptor. EGF receptor was purified from fresh term human placenta as described in the legend to Fig. 1. The receptor was digested with trypsin and peptides separated by reverse phase HPLC as described above. Peptides in the column breakthrough are not shown.

Amino acid sequence

Receptor was purified by immunoaffinity chromatography followed by either preparative SDS gel electrophoresis or by gel permeation HPLC in guanidine⁴³ after reduction and alkylation⁴⁴ to cleave disulphide bonds (see Fig. 1). Purified receptor was then digested with trypsin or cyanogen bromide (see Figs 2 and 3) and peptides were separated by preparative reverse phase HPLC^{45,46} (Fig. 3). Amino acid sequences were determined with a gas phase sequencer constructed and operated as described by Hewick *et al.*⁴⁷, using the analytical techniques for the quantitation of phenyl thiohydantoin (PTH) amino acids described by M.D.W. *et al.*⁴⁸. The quantitative data for analysis of six peptides are shown in Fig. 4.

The amino acid sequences of 14 different peptides from the human EGF receptor, three from placenta and 11 from A431 cells, were compared with sequences in an oncogene sequence data base (set up at ICRF using published sequences) by the rapid search techniques of Wilbur and Lipman⁴⁹. A remarkable identity was found between the sequences of six of these peptides and regions of the predicted sequence of the putative transforming protein *v-erb-B* of the AEV-H isolate of avian erythroblastosis virus³⁰. Of the 83 amino acid residues from these six sequenced peptides, 74 residues were identical and four showed conservative substitutions when they were aligned with the *v-erb-B* encoded protein sequence, as shown in Fig. 5. Peptide 1 was located near the amino terminus of the *v-erb-B* protein (residues 107–125) and peptide 6 at the carboxy terminus (residues 583–599), with the other four peptides in between. It was not necessary to introduce any deletions or insertions into the sequence to optimize the alignments.

Although the full extent of the similarity between the *v-erb-B* protein and EGF receptor sequences is not revealed by these limited sequence studies, it is likely that the region of the *v-erb-B* protein from residue 107 to the carboxy terminus has extensive homology to the EGF receptor. The degree of identity observed is very high and since the *v-erb-B* sequences of AEV were presumably of avian origin³⁰, while the EGF receptor sequences were from the human protein, it is likely that the *v-erb-B* sequences were mainly acquired by AEV from those cellular sequences which encode the avian EGF receptor. This suggests that the *c-erb-B* locus encodes the EGF receptor in humans and birds.

The amino acid sequence of 8 of the 14 peptides purified from the EGF receptor (data not shown) could not be aligned with the predicted sequences of the *v-erb-B* protein. Since the polypeptide backbone of the EGF receptor glycoprotein is thought to be about 1,250 amino acids⁵⁰ and the predicted *v-erb-B* protein is only 604 amino acids³⁰ the most likely explanation is that these eight peptides are encoded by a region of *c-erb-B* which has not been acquired by AEV. This could have arisen by a recombination event(s) which resulted in only a part of the EGF receptor coding sequences being acquired by AEV. Although it is possible that DNA rearrangements of receptor coding sequences occur similar to those found with immunoglobulins, it is more likely that differential mRNA splicing would be involved in any such recombination events. It has been shown that avian cells contain two *c-erb-B* related transcripts⁵¹ and studies of the biosynthesis of the EGF receptor in A431 cells suggest that both a normal and a truncated receptor may be synthesized⁵⁰. Alternatively two or more loci encoding polypeptides having very similar amino acid sequences to those of the EGF receptor exist on chromosome 7 (see below). An example of two closely related putative transforming proteins with tyrosine kinase activity has been reported in studies of the avian retroviruses Rous sarcoma virus (RSV) and Yamaguchi Y63⁵². The predicted amino acid sequences of the proteins encoded by the *src* and *yes* oncogenes were shown to have 82% match over a region covering 436 amino acid residues (while the DNA sequences showed only 31% overall match) and presumably the chicken genome contains both *src* and *yes* proto-oncogenes encoding separate proteins sharing extensive regions of sequence. It is not known whether human *c-src* and *c-yes*

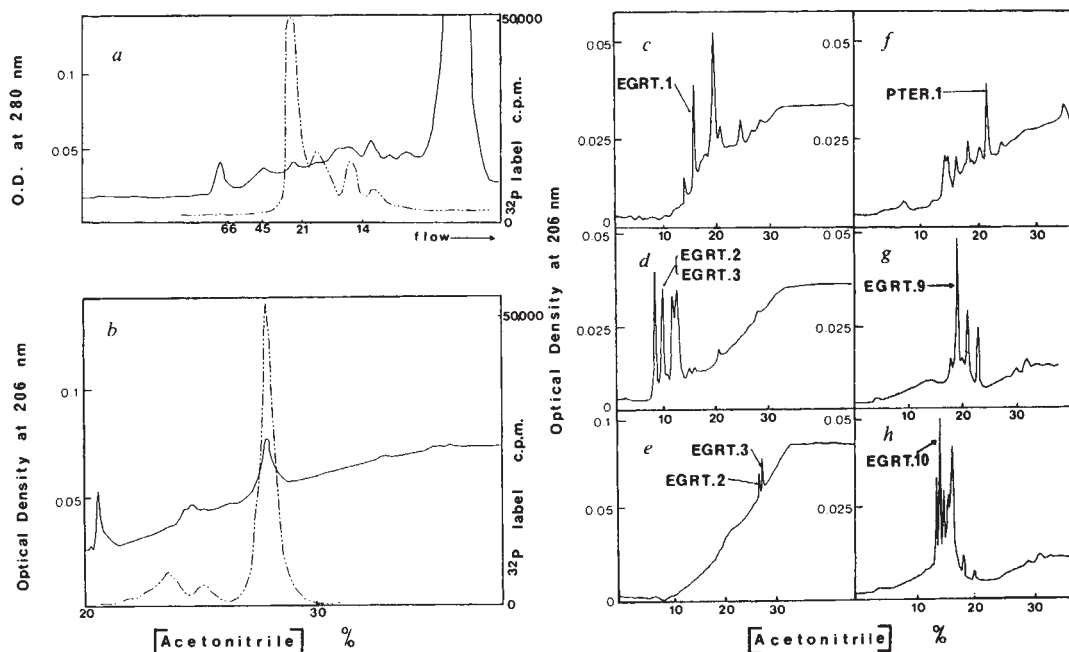


Fig. 3 Purification of peptides from EGF receptor for sequence analysis. **a**, Cyanogen bromide cleavage and fractionation of peptides by size. ^{32}P -labelled EGF receptor in ammonium bicarbonate solution was lyophilized and resuspended in 70% formic acid. Cyanogen bromide was added under nitrogen, the tube sealed and incubated in the dark at room temperature for 24 h. Formic acid and excess cyanogen bromide were removed by repeated cycles of drying and resuspension in water using a Speed-vac concentrator (Savant). The dry sample was resuspended in 0.1 M potassium dihydrogen phosphate buffer, pH 4.5 containing 6 M guanidine HCl and the peptides separated by gel permeation HPLC on a TSK3000 column (0.7×60 cm, LKB) equilibrated in the same buffer at a flow rate of 0.3 ml min^{-1} . The optical density of the eluate was monitored at 280 nm (---) and 0.3 ml fractions collected and counted for ^{32}P (- - -). Molecular weights $\times 10^{-3}$ of protein standards are indicated. **b**, Subfractionation of cyanogen bromide fragments. The peak from the TSK3000 column containing most of the ^{32}P -label was pooled and dialysed against 10 mM ammonium bicarbonate. After lyophilization, the sample was redissolved in 0.1% TFA and peptides separated by reverse phase HPLC on a Synchropak RPP C18 column (see Fig. 2) equilibrated in 0.1% TFA, 10% acetonitrile^{45,46}. A gradient of 10–40% acetonitrile run over 60 min was used to elute peptides, at a flow rate of 1 ml min^{-1} . The optical density of the eluate was monitored at 206 nm (---) and 1 ml fractions were collected and counted for ^{32}P -label (- - -). **c**, The fractions corresponding to 23–24% acetonitrile from the HPLC analysis of A431 EGF receptor tryptic peptides were pooled. Peptides were further purified by reverse-phase HPLC on a Synchropak RPP C18 column equilibrated in 10 mM ammonium acetate buffer pH 6.5. A linear gradient of 0–45% acetonitrile was run over 45 min at a flow rate of 1 ml min^{-1} . The optical density of the eluate was monitored at 206 nm and 0.5 ml fractions collected. **d**, The fractions corresponding to 19–20% acetonitrile from the reverse-phase HPLC purification of A431 EGF receptor tryptic peptides (Fig. 2a) were pooled. Peptides were separated as described in **c**. **e**, The peak fractions arrowed in **d** were pooled and peptides subfractionated by reverse-phase HPLC on a μ Bondapak phenyl column (0.46×25 cm, Waters Assoc.) equilibrated in 0.1% TFA. A linear gradient of 0–45% acetonitrile over 45 min was used to elute peptides, at a flow rate of 1 ml min^{-1} . The optical density of the eluate was monitored at 206 nm and 0.2 ml fractions collected. **f**, The fractions corresponding to 27–28% acetonitrile concentration from the reverse-phase HPLC analysis of placental EGF receptor tryptic peptides (Fig. 2c) were pooled. Peptides were subfractionated as described in **c**. **g**, The fractions corresponding to 25–26% acetonitrile from the reverse-phase HPLC purification of A431 EGF receptor tryptic peptides (Fig. 2a) were pooled. Peptides were subfractionated as described in **c**. **h**, The fractions corresponding to 21–22% acetonitrile from the reverse-phase HPLC analysis of A431 EGF receptor tryptic peptides (Fig. 2a) were pooled. Peptides were subfractionated as described in **c**.

are encoded by closely linked loci. However, analysis of human-mouse somatic cell hybrids has shown that the locus encoding the human EGF receptor is on chromosome 7 (7p13-7q22)^{53–55} and that for *c-erb-B* is in the same region of this chromosome (7pter-7q22)⁵⁶. This observation supports the concept that the *c-erb-B* locus encodes the human EGF receptor or is very closely linked to that encoding the EGF receptor.

Analysis of EGF receptor mRNA transcripts found in A431 and avian cells by cDNA cloning is currently in progress which, together with analysis of human and avian genomic clones, should define the relationship of *c-erb-B* and the locus encoding the EGF receptor (A.U. *et al.*, in preparation).

Shared regions of sequence

Several lines of evidence suggest that the EGF receptor protein can be divided into three major domains; an EGF binding domain which lies external to the plasma membrane, a trans-membrane domain and a cytoplasmic kinase domain having both the kinase activity and the autophosphorylation sites.

Investigations of receptor biosynthesis show that the A431 receptor is a glycoprotein of apparent molecular weight (MW) 175,000, having $\sim 37,000$ MW of oligosaccharide side chains with a polypeptide backbone of $\sim 138,000$ MW ($\sim 1,250$ amino acids). Limited proteolysis of the mature receptor suggests that the domain external to the plasma membrane which contains

the oligosaccharide side chains and the antigenic sites for monoclonal R1 has a MW of $\sim 115,000$ (~ 710 amino acids)⁵⁰. Several studies show that the EGF binding site is external to the plasma membrane^{25,31,32}.

The location of the tyrosine kinase enzymatic activity and the autophosphorylation sites on the cytoplasmic domain is supported by studies made using A431 and placental membrane vesicles (J.D. and M.D.W., in preparation). These show that EGF stimulated tyrosine kinase activity directed towards artificial substrates or towards autophosphorylation sites is significantly activated only after membrane permeabilization. Furthermore, the tyrosine kinase activity can phosphorylate pp36—a protein known to be located at the cytoplasmic side of the membrane⁵⁷. In addition recent studies show that antibodies raised against synthetic peptides from pp60^{v-src} recognize antigenic sites on the human EGF receptor that are from regions of sequence homologous to the sequence of *v-erb-B* (see below). These sites are only accessible in permeabilized cells (J.S. *et al.*, in preparation).

Autophosphorylation sites are located within peptide 5 (EGRC.1), a 20,000 MW cyanogen bromide fragment which contains 70% of the ^{32}P -label present in the autophosphorylated receptor (see Fig. 3a). Although the precise location of the residues phosphorylated has not been determined a consensus tyrosine phosphorylation sequence⁵⁸ was found near the amino

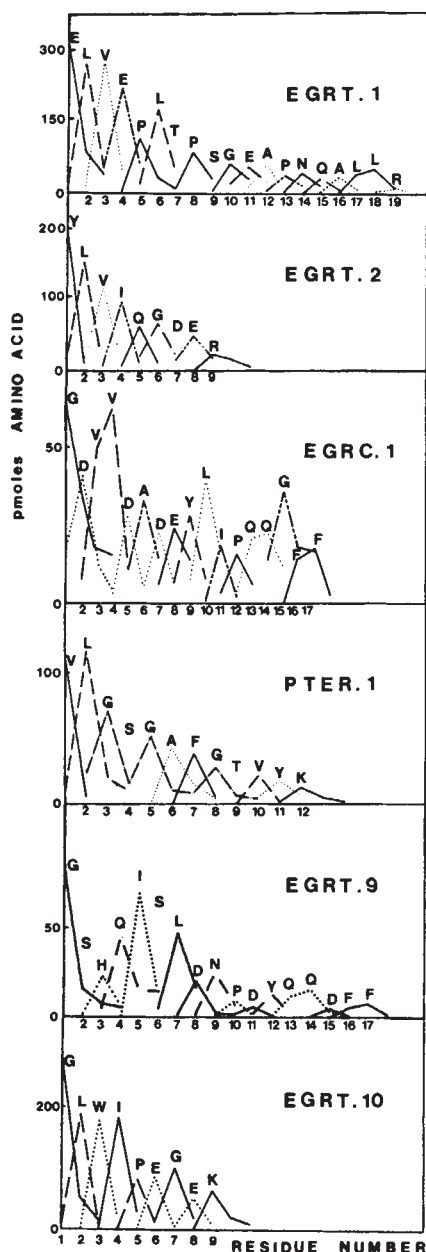


Fig. 4 Sequence analysis of peptides from the EGF receptor. Peptides were purified as described in Fig. 3. Sequence determination of each peptide was carried out using a gas phase sequencer assembled and operated as described by Hewick *et al.*⁴⁷. PTH amino acids were analysed by HPLC using a Zorbax C8 column (4.6×150 mm, Dupont) at 43 °C with a linear gradient over 8 min of acetonitrile from 24% to 38% at a flow rate of 2 ml min⁻¹ using 9 mM sodium acetate buffer pH 4.1⁴⁸. A Waters HPLC system including two M6000 A pumps, a WISP autoinjector and system controller with a Beckman Model 160 detector was used. The recovery of PTH amino acids at each degradative cycle was measured using an integrative recorder (Waters Data module). The amounts of each peptide analysed (pmol) were measured by amino acid analysis (EGRT.1, 380; EGRT.2, 260; EGRC.1, 80; PTER.1, 145; EGRT.9, 100; EGRT.10, 325). The analysis for serine and threonine could not be accurately measured due to the presence of multiple peaks obtained during analysis of the PTH amino acids. The presence of these amino acids is thus indicated without quantitative data; these residues are assigned to the sequence using semi-quantitative recovery data based on peak heights rather than areas. Prior to loading peptides, fibre glass disks were treated with polybrene and glycylglycine and precycled for ten cycles. Each peptide was sequenced twice; on the second run of peptide EGRC.1 filters were treated with polybrene and cysteic acid and precycled ten times to clarify the assignment of an amino terminal glycine residue. However the background glycine at step 1 is still significant and this residue may be incorrect.

terminus of this peptide. Therefore we believe that the tyrosine phosphorylation sites lie within the cytoplasmic domain of the EGF receptor which is contained in the sequence shared with the *v-erb-B* protein.

Preliminary nucleotide sequence analysis (A.U. *et al.*, in preparation) of cDNA clones selected from a placental cDNA library using synthetic oligonucleotide probes synthesized on the basis of the receptor amino acid sequence shows that the predicted carboxyl terminus of the EGF receptor extends 32 amino acids from an amino acid equivalent to residue 601 (see Fig. 5) of the predicted *v-erb-B* protein sequence. This analysis when complete will show the precise size and sequence of the presumptive cytoplasmic domain of the EGF receptor. The approximate molecular weight of this domain would be 60,000 (or 545 amino acids) since that part which is external to the membrane is thought to have a molecular weight of 115,000 (see above)⁵⁰. Thus the cytoplasmic domain would be predicted to be similar in size to that region of the *v-erb-B* protein which is carboxy terminal to a putative transmembrane sequence (see Fig. 5 and ref. 30) at residues 66–88. This carboxy terminal region of *v-erb-B* would have a molecular weight of 56,760 and would contain 516 amino acids.

The putative transmembrane sequence of the *v-erb-B* protein is not preceded by a signal sequence for membrane insertion. Nevertheless, immunofluorescence studies of AEV transformed cells show that the *v-erb-B* protein has antigenic sites external to the plasma membrane⁵⁹. This external region probably corresponds to the 65 residue amino terminal section that precedes the putative transmembrane sequence and contains three asparagine residues which have the oligosaccharide attachment recognition sequence Asn-X-Ser or Thr. Some or all of these residues may be glycosylated since *in vitro* translation studies of mRNA from AEV infected cells show that post translational processing of nascent polypeptides occurs in the presence of membrane vesicles^{59,60}.

Together these studies suggest that the predicted *v-erb-B* transforming protein closely resembles the transmembrane region of the EGF receptor and the domain which is thought to be cytoplasmic. If the *v-erb-B* sequence was acquired from the gene encoding the EGF receptor then the *v-erb-B* protein represents a truncated receptor which lacks the EGF binding domain. It is particularly interesting that studies of EGF receptor biosynthesis in A431 cells have suggested that a polypeptide equivalent to the external domain of the receptor (of MW 115,000) is synthesized⁵⁰ in addition to the normal receptor. Further studies are necessary to understand the origin of this truncated receptor but the results show that defective receptors may be synthesized by this human tumour cell line.

Growth factors and transformation

Recently it has been shown that the transforming protein of simian sarcoma virus has a close structural and functional relationship to the growth factor PDGF^{7–10} supporting the hypothesis that autocrine growth factor production may be involved in abnormal growth control and neoplasia. These observations together with those presented here illustrate two distinct but related mechanisms for subversion of normal growth regulation. In the case of SSV the oncogene encodes a growth factor which can act as a mitogen for target cells having PDGF receptors¹⁰. AEV on the other hand appears to utilize a different mechanism where a part of a growth factor receptor which is thought to be involved in transducing the EGF signal may be expressed in transformed cells. The absence of the EGF binding domain might remove the control generated by ligand binding and the result could be the continuous generation of a signal equivalent to that produced by EGF, causing cells to proliferate rapidly. How this could result in the block in differentiation observed in AEV infected haemopoietic cells⁶¹ is unclear. However, EGF has been shown to promote proliferation while inhibiting terminal differentiation of human keratinocytes³¹.

The ES4 strain of AEV has two oncogenes *v-erb-A* and *v-erb-B*, which are thought to encode proteins of MWs 75,000

Src 1 MGSSKSKPKDPSQRHSLPEPDSHGGFASQSTPDETAAPDAHRNPSRS
Src 51 FGTVATEPKLFWGNTSDTVTSQRAGALAGGVTTFVALYDYESHTETDL
Src 101 SFKKGERLQIVNTEGDWHLAHSLLTGQTGYIPSNYVAPSDSIQREWHYF
 MKCAHFIDGPHCVKA
Src 151 GKITRRESERLLNPNPAGTFLVRKSETAKGAYCLSYSDFNAGKPNVK
Erb-B 16 CPAGVLGENDTLVRKYADANAVCOLCHPNCTRGCKGPGLEGCPNGSKTFS
 TAAAGVVGGLLCLVVGGLGIGL YLRRRHIVRKRTRLRLLOERLEVEPLT-P
Src 201 HYKIYKLYSGGFYITSRTOFGSLQOLVAYYSKHAQGLCHRLANVCPTSKP
Erb-B 66 TAAAGVVGGLLCLVVGGLGIGL YLRRRHIVRKRTRLRLLOERLEVEPLT-P
Src 251 QTQGLAKDAWEIPRESLRLEAK-LGGGCFGEVHMOTHN---D---TTRVAI
Erb-B 115 GEARPNQAHRLIKETEFKKVKVLGSGAFDTIYKGLHIFEGEKYKIPVAI
 BGEARNQALLL VLGGGAFDTIYKGLHIFEGEKYKIPVAI
Src 295 KILKPGTMSK---FLOEGVVMKSLMEKLVOLYAVVSEEPYIVIEYH
Erb-B 163 KILREAT-SFKANKEILDERYVMSVDNPHVCGLLGICLTSTVGLITGLH
Src 346 SKGSLDLFLKGMCKYLRLLPQ-LVDMARQISQMHAYVE-RMNYVHADLRA
Erb-B 214 PYGCLLDY-IRE-HKDNIGSGYLLNLCVQIAKGMNYLEARR-LVHADL-A
Src 390 A-NILVGENLVCKYADFLALRIE-D-NEYTARQAKFIKHTAPEARLY
Erb-B 260 ARNVLKVTQGHVKITDFGLAKLLGARKEVHREGG-KYPIKMALESILH
Src 437 GRFTIKSDVHFGILLTELTKGRVYPMVNVREGLDVERGYMPCPPE
Erb-B 389 RIYTHQSDVMSYQVYVHLMFTGSKPYDQIPASEISSVLEKGERLPGPPI
Src 487 CPESLHLMCOCHRDPEERPTFKYLORQLLPRACVLEVE
Erb-B 359 CTIDVYIMVYKCMHIDRDSNPKPRELIEAFSKMARDPPRYLVIGQDERMH
 *VLVIGQDERMH
Erb-B 489 LPSPTDSKFYRTLMEEDMEDIVDADEYLVPHGGFFNSPSTSRTPLLSSL
 *GVVDADDEYLVPHGGFF
Erb-B 459 SATSNNSATNCIDRNGQGHVREDSDVQRYSSDPTGNFLEESIDGFLPA
Erb-B 589 PEYVNLMPKKPSTAMVQNGIYNFISLTAISKLPMSRYQNSHSTAVDNP
Erb-B 559 EYLNTNQSPAKTVFESSPYWISQGNHNLNPNPYGQDFLPTSCS
 *MSHQLSLNPNPYGQDF

Fig. 5 The relationship between the amino acid sequences of the EGF receptor peptides and the predicted amino acid sequences of the putative transforming proteins of *v-src* and *v-erb-B*. The predicted amino acid sequence of the *v-src* gene product (pp60^{v-src}) is translated from the presumptive initiation codon at nucleotide 7,129 of the Prague C strain of Rous sarcoma virus⁷³. The predicted amino acid sequence of the *v-erb-B* gene product is translated from the presumptive initiation codon at nucleotide 155 of the *v-erb-B* gene in AEV-H³⁰. The partial amino acid sequences of the six peptides purified from the EGF receptor are shown (underlined): 1, EGRT.1; 2, PTER.1; 3, EGRT.10; 4, EGRT.2; 5, EGRC.1; 6, EGRT.9. Letters in bold type represent residues shared by pp60^{v-src} and the *v-erb-B* protein. Residues shared by the EGF receptor peptides and *v-erb-B* protein or pp60^{v-src} are in bold type. ● indicates amino acid residues, which are common to the putative transforming proteins of *v-erb-B*, *v-src*, *v-fes*, *v-fps*, *v-yes* and *v-abl*. †, Phosphoacceptor tyrosine of pp60^{v-src}⁷⁴. ▲, Possible N-linked glycosylation sites at the amino terminus of the *v-erb-B* protein. — indicates the putative transmembrane sequence in the *v-erb-B* protein. *, Amino acid residues which would be expected to produce enzymatic or cyanogen bromide cleavages to generate the observed peptides. Numbers to the left of the sequences are residue numbers taking the presumptive initiation methionine as 1 in both cases. Sequences were aligned using a computer program⁷⁵ to optimize match.

and 65,000 respectively (for a recent review see ref. 62). Cells transformed by AEV *in vitro* and *in vivo* have the properties of erythroblasts which are late erythroid progenitors, although the target cells themselves may be earlier erythroid precursor cells. AEV can also transform fibroblasts and induce sarcomas. Evidence from deletion mutants⁶³ and from an isolate (AEV-H³⁰) which lacks the *v-erb-A* gene suggests that the *v-erb-B* gene alone can induce transformation. This is supported by studies which show that RAV-1, a leucosis virus which has no oncogene, can activate the *c-erb-B* gene by a promoter insertion mechanism⁶⁴ perhaps similar to that presently being unravelled for *c-myc* activation⁶⁵⁻⁶⁷. It is possible that RAV-1 could induce expression of a normal receptor or a truncated receptor. EGF receptors have not generally been detected on haemopoietic cells by EGF binding studies but since these studies are limited in scope and sensitivity a more rigorous survey is needed before conclusions about normal receptor expression in different haemopoietic cell types can be made. Although many normal

cells express 10–100,000 EGF receptors³¹ only very low levels of *c-erb-B* transcripts have been found in normal chicken fibroblasts⁵¹. However a recent study of normal and neoplastic human lymphocytes suggests that both types of cells contain *c-erb-B* related transcripts. Clearly more detailed studies will be necessary to search for normal or altered *c-erb-B* or *v-erb-B* transcripts in normal, neoplastic or AEV transformed cells.

Previous reports have shown that the predicted amino acid sequences of the putative viral transforming proteins encoded by the oncogenes *erb-B*, *src*, *yes*, *fes*, *fps*, *mos* and *abl* show regions of similarity, implying homology^{29,30,69}. In the case of *src*, *yes*, *fes*, *fps* and *abl* the putative transforming proteins have been shown to have tyrosine kinase activity (reviewed in ref. 29) but as yet those encoded by *erb-B*³⁰ and *mos* have not. Since the receptors for EGF and αTGF, PDGF, insulin and IGF-I also have associated tyrosine kinases, the structural relationship between the *v-erb-B* transforming protein and the EGF receptor observed here, suggests that other oncogenes from this subset of retroviruses could be derived in part from sequences encoding these or other growth factor receptors. Alternatively the other oncogenes in this group may be derived from sequences which encode proteins having a less direct functional relationship to the EGF and other receptors. Further study will clarify the precise function of growth factors, their receptors and *src* related oncogenes in normal and malignant growth.

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LETTERS TO NATURE

Interstellar scintillation and ultra-low-frequency gravitational wave observations

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High-precision pulsar timing measurements have been used¹⁻⁴ to set upper limits on the flux of ultra-long period ($T \geq 3$ years) stochastic gravitational waves. The data used to produce the current limits appear to be limited by timing noise^{5,6} that is intrinsic to the pulsars. Recently, however, there have been very low noise timing observations⁷ of the 1.5-millisecond pulsar⁸. This suggests that significant sensitivity improvements in searches for ultra-low-frequency (ULF) gravitational waves may be possible. Here the effects of a heretofore secondary noise source—phase scintillations caused by interstellar electron density fluctuations—are calculated and the prospects for improved ULF gravitational wave measurements using pulsar timing are discussed.

The observed quantity in these experiments is a time series of the difference between the observed and expected pulse arrival times. The time series contains contributions from intrinsic timing noise, gravitational radiation (buffeting of the pulsar and Earth at different epochs), estimation error due to finite signal to noise ratio, and interstellar phase scintillation (ISS). The ISS contribution arises due to dispersion measure fluctuations as irregular clouds of electrons rearrange and drift across the pulsar–Earth line^{9,10}. To estimate the contribution to pulsar timing noise measurements a model of the interstellar turbulence spectrum and the relevant propagation theory are required. Observations¹⁰⁻¹² are consistent with an isotropic three-dimensional electron density power spectrum, $P_{3N}(q)$, of the form $P_{3N}(q) = C_N^2 q^{-11/3}$ where q is the spatial wavenumber. The 'structure constant' C_N^2 is typically 10^{-5} to $10^{-2} \text{ m}^{-6.67}$ (ref. 11), but can be estimated for the line-of-sight to the 1.5-millisecond pulsar from the measured frequency correlation bandwidth in the dynamic spectrum⁸ or the measured temporal broadening of pulses¹³. Using the observed correlation bandwidth ($\approx 1 \text{ MHz}$ at $\lambda = 0.21 \text{ m}$ observing wavelength) and the observed pulse broadening ($73 \mu\text{s}$ at $\lambda = 0.94 \text{ m}$), C_N^2 can be estimated using equations 31 and 41 of ref. 10. For homogeneous turbulence along the pulsar–Earth path of 5 kpc (ref. 14), those

observations yield identical estimates of $C_N^2 = 8 \times 10^{-5} \text{ m}^{-6.67}$. Because both decorrelation bandwidth and pulse broadening are diffraction phenomena, those observables are dominated by density fluctuations on scales of the Fresnel zone size ($\sim 10^{10} \text{ m}$). However, direct and indirect measurements of P_{3N} on the 10^{11} – 10^{13} m scales relevant to ULF gravitational wave measurements are consistent with an extrapolation of the power at smaller scales; the inferred shape and level of the spectrum on these scales is in fairly good agreement with $P_{3N}(q) = (10^{-4} \text{ m}^{-6.67}) q^{-11/3}$ (ref. 11).

For gravitational wave searches, noise sources can be characterized either by the power spectrum of fractional frequency fluctuations^{2,15} or the Allan variance¹⁶ of the frequency time series¹⁷. The spectrum of fractional frequency fluctuations caused by ISS, $S_y(f)$, can be calculated in three steps. First, ignoring diffraction at the relatively large scales of interest here, the two-dimensional spatial spectrum of phase on the Earth's surface is given by (ref. 9)

$$P_{2\phi}(\kappa) = 2\pi\lambda^2 r_e^2 z C_N^2 \kappa^{-11/3} \quad (1)$$

where κ is the two-dimensional wavenumber, λ is the observing wavelength, r_e is the classical electron radius, and z is the pulsar–Earth distance. Second, to convert to a temporal spectrum of phase fluctuations it is assumed that the phase scintillation pattern is 'frozen' in a reference frame that moves with a uniform velocity v transverse to the pulsar–Earth line. Integrating over the wavenumber component associated with the direction perpendicular to the velocity vector then gives the temporal phase spectrum. Finally, noting that frequency is the temporal derivative of phase, the 'derivative theorem' for Fourier transforms¹⁸ can be applied to obtain $S_y(f)$. The result is:

$$S_y(f) = \pi^{-1/6} 2^{-2/3} v^{5/3} \lambda^4 c^{-2} z C_N^2 r_e^2 [\Gamma(4/3)/\Gamma(11/6)] f^{-2/3} \quad (2)$$

where c is the speed of light, v is the velocity of the pattern, Γ is the gamma function and f is the temporal frequency.

Equation (2) can be used to predict ISS noise for ULF gravitational wave observations. Figure 1 shows the current observational limits to a ULF gravitational wave background²⁻⁴, the envelope of stochastic gravitational wave spectra having closure density¹⁹, and plots of equation (2) evaluated with the following parameters: $\lambda = 0.21 \text{ m}$ and 0.06 m , $z = 5 \text{ kpc}$, $C_N^2 = 8 \times 10^{-5} \text{ m}^{-6.67}$, $v = 100 \text{ km sec}^{-1}$. Also shown in Fig. 1 is the $S_y(f)$ that would be observed for daily observations of a pulsar having white intrinsic timing noise with spectral density $9 \times 10^4 \mu\text{s}^2 \text{ Hz}^{-1}$. That is, the normalization is set by $1 \mu\text{s}$ (r.m.s.) white timing noise distributed over the Nyquist band¹⁸ 0–0.5 cycles d^{-1} set by the daily sampling interval.

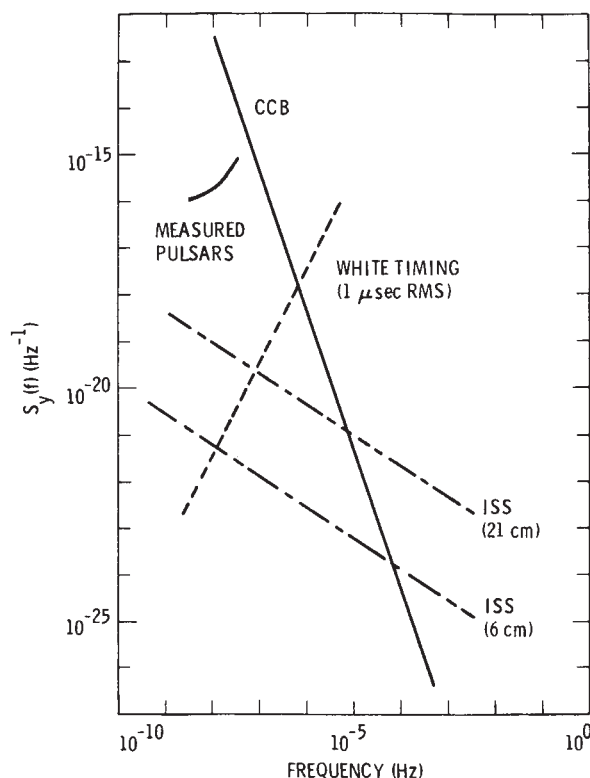


Fig. 1 Plot of power spectrum of fractional frequency fluctuations, $S_y(f)$, as a function of fluctuation frequency, f . Curve labelled 'CCB' is envelope of cosmic gravitational wave background spectra having closure density¹⁹; curve labelled 'Measured Pulsars' is observed limit to S_y from Hellings and Downs² and Romani and Taylor³; curves labelled 'ISS' are spectra expected due to interstellar scintillations (parameters: $\lambda = 0.21$ m and 0.06 m, $C_N^2 = 8 \times 10^{-5} \text{ m}^{-6.67}$, $z = 5$ kpc, $v = 100 \text{ km s}^{-1}$); curve labelled 'White Timing' is for white intrinsic pulsar timing noise with r.m.s. value of 1 μs in the band 0–0.5 cycles d^{-1} .

From Fig. 1, the ISS limit is about three orders of magnitude below the current observational limit to S_y at $f = 10^{-8} \text{ Hz}$. However, as the intrinsic timing noise becomes lower ISS becomes increasingly important; white 1 μs r.m.s. timing noise becomes ISS-noise dominated at periods ~ 4 months for 21 cm observations. Expressed as a fractional frequency stability, the 21 cm ISS-curve in Fig. 1 corresponds to an Allan variance of $\approx 1.5 \times 10^{-14} (\tau/1 \text{ yr})^{-1/6}$. (It should be noted that plasma phase scintillations are also an important noise source for gravitational wave observations in the 10^{-1} to 10^{-4} Hz frequency range¹⁵. Here fluctuations in the interplanetary plasma cause perturbations in the radio tracking record of a distant spacecraft¹⁷. The sensitivities achievable with an X-band tracking link are expected to be $\sim 5 \times 10^{-15}$ at $\tau = 1,000$ s integration times for the raw data. Since the spacecraft coherently transponds the signal transmitted from the earth, a gravitational wave produces a characteristic three-pulse response¹⁵ in the tracking time series. Thus correlation analysis should allow further improvement in the sensitivity in the spacecraft tracking case.)

What are the prospects for reducing the ISS noise? Since S_y is proportional to λ^4 , observations at shorter wavelengths rapidly reduce ISS noise. However, pulsar signal-to-noise ratio considerations may make high frequency observations impractical. Dual frequency observations could be used to estimate and remove these dispersion measure fluctuations (ref. 20 for example). Although only changes in the dispersion measure are relevant here, the complicating factors of frequency-dependent pulse shape and frequency-dependent pulse broadening due to ISS diffraction will still make observations difficult at the level of precision required for a good ISS calibration. Without higher frequency observations or high-precision dispersion measure monitoring, the ISS-noise in Fig. 1 will constitute a limit to the

sensitivity of ULF gravitational radiation observations even using timing data from an intrinsically noiseless pulsar.

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Surface albedo and the Sahel drought

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The persistence of the Sahel drought, which reached a peak in 1973, appears to be typical for such dry episodes over past decades and centuries^{1–3}. Such strong persistence can be understood if a strong positive feedback mechanism is operating, partly driven by changes in surface properties^{4,5}. The key factors in the mechanisms thus far studied are the surface albedo and the soil moisture, both of which affect the radiation balance at the surface, the first directly, the second indirectly through its influence on the latent heat flux. We have now studied the evolution of the albedo of this region since 1972. We find that dry season albedo in the Sahel (notably Ferlo and Gondo regions, see Fig. 1b) declined from a maximum close to 0.30 in 1973 to values close to 0.20 in 1979. This decline is consistent with changes in plant cover determined by analysis of spectral changes in the Landsat multispectral scanner (MSS) data and field studies^{6,7}.

The effect of drought, reducing soil moisture and thus evaporation and cloud cover, and increasing surface albedo as plant cover is destroyed, is generally to increase ground and near-surface air temperatures while reducing the surface radiation balance and exacerbating the deficit in the radiation balance of the local surface-atmosphere system. This entails increased atmospheric subsidence and consequently further reduced precipitation. The biogeophysical feedback mechanism involving albedo was first proposed by Charney⁸, who noted that to the extent that man's agricultural and pastoral activities increased the stress on plant cover, they might be a factor in the increase of surface albedo, thus contributing to the persistence of the Sahel drought. Various numerical studies have confirmed that very large increases in surface albedo should indeed lead to substantial reduction in precipitation^{9–12}, but the question remains whether these assumed changes in surface albedo bear

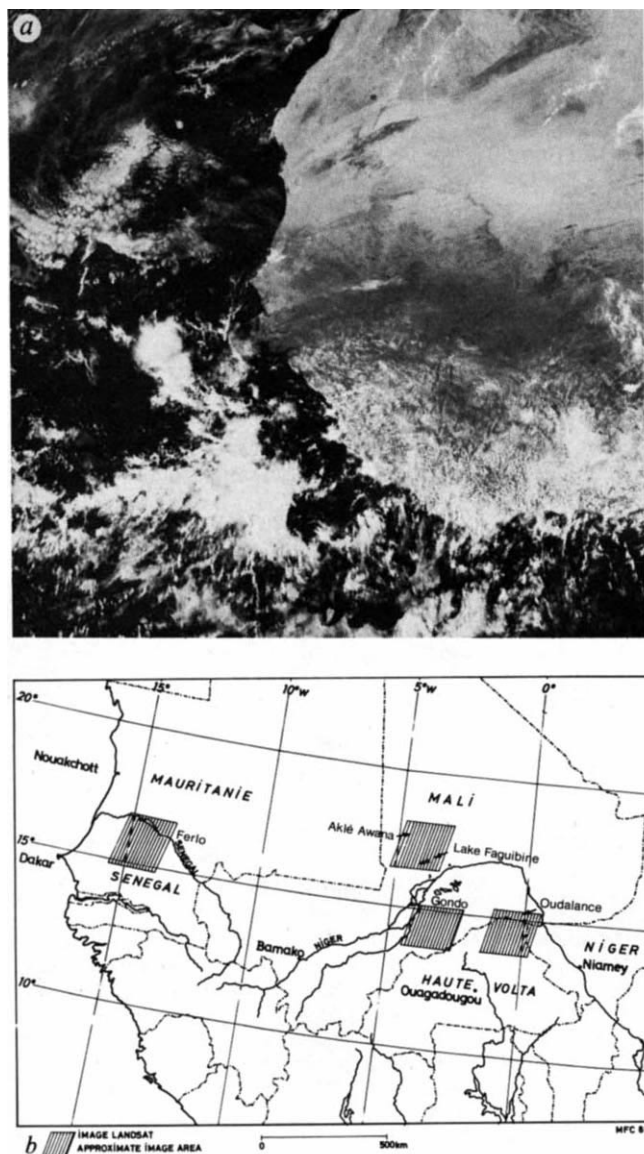


Fig. 1 West Africa: Saharo-Sahelian area. *a*, Portion of the Meteosat visible channel midday image of 2 August 1979. *b*, Map showing areas observed with Landsat; from west to east these are: Ferlo (Senegal), Aklé Awana with Lake Faguibine (Mali), Gondo (Mali), Oudalance (Haute-Volta).

any relation to those which have occurred in reality. For the period 1967–74 when the drought was intensifying, Norton and co-workers¹³ found moderate increase in surface albedo, strong during the wet season months. The limitation that we found to the extension of the albedo, despite the continued relatively dry conditions, may result from accumulation of plant debris¹⁴ (in regions such as Ferlo protected from overgrazing) and/or rapid recovery of grass cover even when the total amount of living vegetation continues to decrease. Thus the Charney albedo mechanism may not have a major role in the long-term evolution of the Sahel climate.

Our analysis considers the western part, from the Atlantic coast to longitude 3° E, of the Saharo-Sahelian zone of Africa, between latitudes 14 and 20° N. Norton and co-workers studied the changes in this region in the period 1967–74 using green sensor data from the Applications Technology Satellite (ATS-3), presenting results for areas 2° square¹³. We have studied the evolution of the region since 1972, using two sets of satellite data: (1) on the larger scale, directly comparable with the ATS-3 data, we use hourly visible channel data from Meteosat-1, for several periods of 1–6 days distributed through the year from

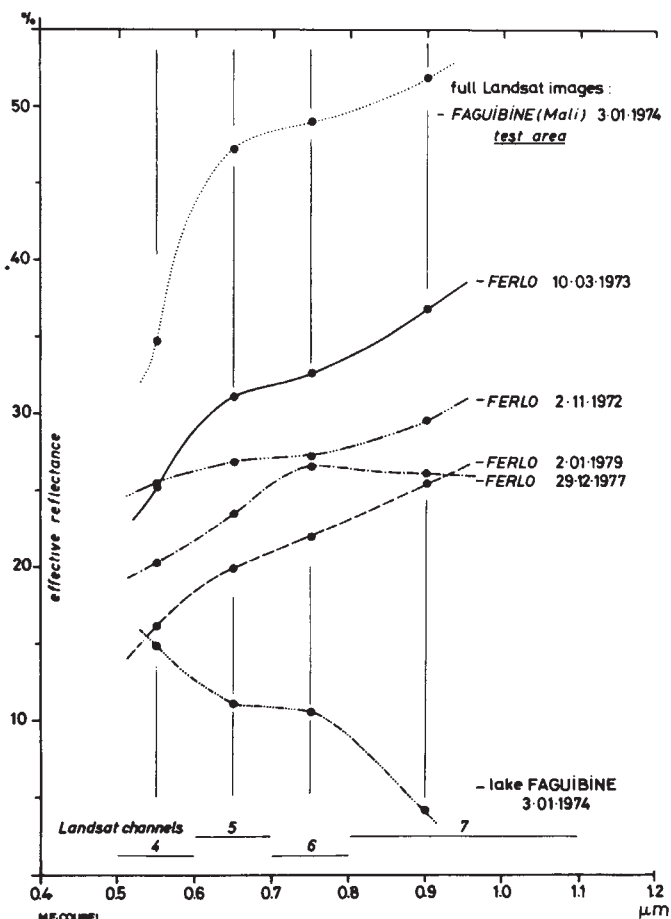


Fig. 2 Spectral reflectance based on Landsat MSS data. The two extreme curves are for the water surface of Lake Faguibine (lowest values) and for the full Landsat image including the lake and made up mostly of sand dunes (Aklé Awana), yielding the highest values.

November 1978 to November 1979. The spatial resolution of $2.5 \times 5 \text{ km}^2$ (somewhat better than ATS-3) has been degraded to $(40 \text{ km})^2$ in preparing albedo maps of the entire region, and then to the 2° squares of Norton *et al.* (2). On a much finer scale, we use digital Landsat MSS data (spatial resolution 79 m) for several areas of $(185 \text{ km})^2$ (see Fig. 1*b*), obtained on a series of dates from 1972 to the present, at about 9.30 LT in each instance.

To estimate the planetary albedo from satellite measurements of emergent radiances, one must consider not only the sensor calibration, but also possible bias associated with limited spectral, angular and temporal sampling¹⁵. To estimate the surface albedo, one must also consider atmospheric effects.

The calibrated relatively-narrow bands of Landsat (Fig. 2) provide quasi-monochromatic radiance values^{16,17} from which we compute the spectral reflectances and the isotropic estimate α_i of the albedo, integrated over the solar spectrum. Results obtained for the water surface of Lake Faguibine (Fig. 2) agree with theoretical computations of the spectral reflectance¹⁸. On the same Landsat images, the spectral albedo of the bright sand dune area of Aklé Awana is found to be quite high (from 0.35 in channel 4 to 0.52 in channel 7), in agreement with the values of 0.45–0.50 found for the solar spectrum-weighted albedo from the Meteosat images. This bright area was also used as a normalization point (with green channel albedo 0.41) in the earlier ATS-3 study.

For the Meteosat study, we have used Koepke's calibration¹⁹ relating raw visible count to the radiance filtered by the Meteosat visible channel. These values are corrected using a 'filter factor' which is determined using spectral reflectance curves deduced from the Landsat MSS data and from laboratory studies of soil and plant samples from the area of interest^{6,7}. This yields a good

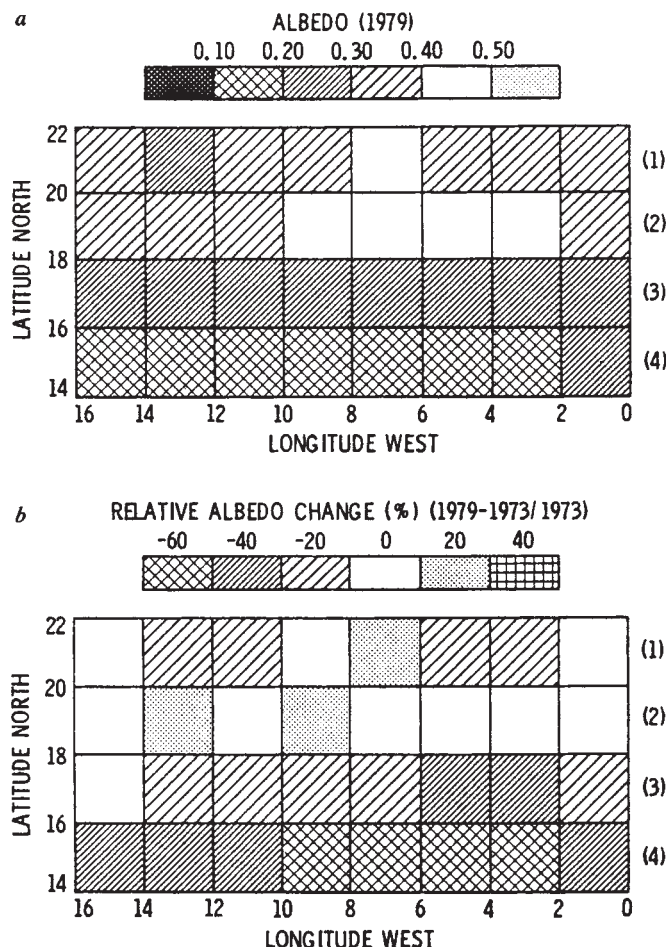


Fig. 3 *a*, Total solar spectrum-weighted surface albedo of the western Sahel, estimated from Meteosat-1 VIS data for 3–5 November 1979, with spatial resolution degraded to 2° . *b*, Relative albedo change $100 \Delta\alpha/\alpha_{1973}$ observed between 19 October 1973 (ATS-3 data) and November 1979.

estimate of the total solar spectrum-weighted reflectance. Also, corrections for anisotropy are small (<0.02 in the instantaneous value of the albedo) because both Meteosat and Landsat are close to the zenith of the areas observed, so that the outgoing direction of greatest weight in determining the albedo is sampled. With the Meteosat data, temporal sampling (solar incidence sampling) is complete: we obtain several albedo estimates per day and find them approximately constant to solar zenith angle 60° over Sahelian and Saharan land surfaces. This suggests that the bias associated with the fixed 9.30 LT of the Landsat observation is fairly small, and even if such bias affects Landsat determinations of an annual albedo cycle, it disappears when comparing observations of different years made at about the same date.

Thus we believe that the various sampling biases in our data are small, or can be accounted for, as far as the planetary albedo values are concerned. The Landsat images used are cloud-free, and judging from reflectance of water surface, turbidity is low. The periods studied with Meteosat were chosen to be relatively cloud-free over the western Sahel, on the basis of visual inspection of both visible and IR images. Days with apparent high turbidity were rejected, and for each 2–3-day period studied, a minimum reflectance was used to estimate cloud-free albedo. Corrections for atmospheric effects (Rayleigh scattering, water vapour and ozone absorption, aerosol scattering and absorption) have been made following the procedure of Otterman and Fraser, and they are small ($<5\%$) in the albedo range of interest^{20–22}. Results are shown in Figs 3 and 4 and discussed further below.

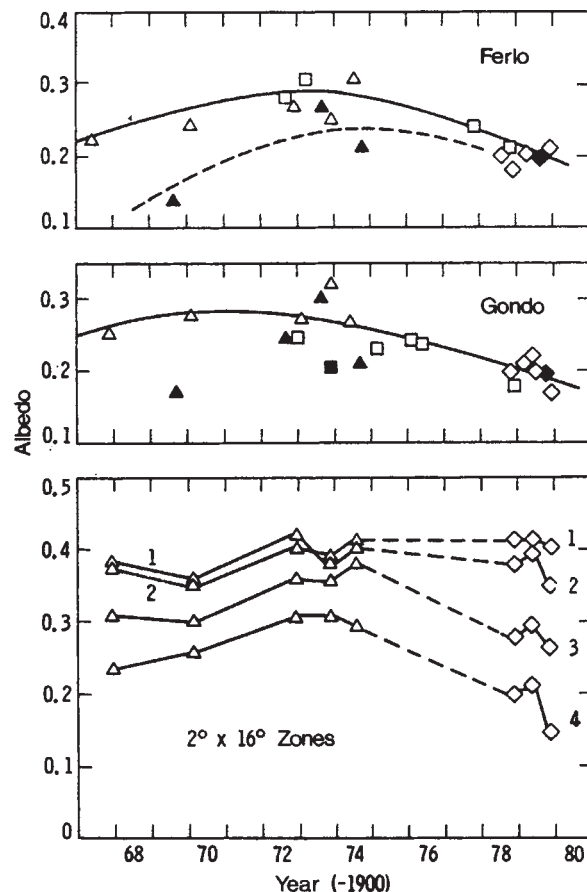


Fig. 4 Albedo history of the western Sahara and Sahel between 1967 and 1979: triangles, estimates based on the 2° maps of Norton *et al.*¹³; squares, Landsat data (Ferlo, Gondo); lozenges, Meteosat-1 data. The filled symbols correspond to observations in the rainy season. Curves 1–4 are integrated albedo values for the four strips 2° wide in latitude, extending from longitude 16° to 0° W, illustrated in Fig. 3.

Both the Landsat and the Meteosat data show that at its brightest spots (for example, Aklé Awana), the Sahara has an albedo close to 0.50. The mean albedo for the region from latitudes 18° to 23° N, and from longitude 10° W to the Greenwich meridian, is 0.42 ± 0.04 , higher than the value of 0.35 used in Charney's model experiment^{8,10}, higher also than the values which often appear in surface albedo compilations²³. The high value is consistent with the results of Otterman and Fraser^{20–22}, who studied a variety of desert sites using both Landsat MSS and laboratory data; it also agrees with some on-site measurements of bright sand in the Empty Quarter of Arabia²⁴. It comes about essentially because of the near-IR rise in reflectance of the surface, observed in channels 6 and 7 of Landsat (Fig. 2).

In the western Sahel, the 1979 Meteosat data indicate effective surface albedos ranging from 0.15 to 0.25 (Fig. 3a). The values for the Ferlo and Gondo regions are in agreement with the Landsat data for the same period (Fig. 4). The earlier Landsat data for these same regions in 1972–73 also agree with values estimated from the ATS-3 $2^\circ \times 2^\circ$ albedo maps. Thus the Landsat data effectively bridge the gap between ATS-3 and Meteosat. For both the Ferlo and Gondo regions, dry season albedo declined from a maximum close to 0.30 in 1973 to values close to 0.20 in 1979 (Fig. 4), following the increase observed between 1967 and 1973. For the western Sahel as a whole, relative decreases of 20–60% were observed between 1973 and 1979 (Figs 3b, 4). This change is noteworthy in comparison with the relative stability ($\pm 15\%$) observed in the Saharan zone (Fig. 3b and curves 1 and 2 of Fig. 4) over the same period, indicating that the two sets of data are comparable.

Only a small fraction of existing satellite data has been examined, and uncertainties in establishing an albedo history are large. We cannot at present evaluate the 'normal' surface albedo of the Sahel, if indeed such a normal exists. However, even the smallest values obtained for the western Sahel (0.20 ± 0.05) and Sahara (0.42 ± 0.04) are larger than the 'climatological means' used in model calculations. Our study also shows that although drought conditions still persist in the Sahel, the surface albedo declined significantly between 1973 and 1979, in contradiction to the expectations of the Charney albedo feedback mechanism.

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Predictability of north-east Brazil droughts

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The droughts of north-east Brazil are unusually well defined in the departures of the quasi-permanent circulation systems in the tropical Atlantic sector. Anomaly patterns in the large-scale atmospheric and oceanic fields evolve in the course of the half-year preceding the March/April rainy season. Based on these diagnostic results, dynamically plausible predictor candidates are selected and entered into a stepwise multiple regression scheme. The circulation in the Atlantic sector proves to be essential for the prediction of rainfall anomalies, while predictor candidates from other parts of the globe are of subordinate importance. The regression equations derived from the 1921–56 record are used to produce rainfall forecasts for the years 1958–72. Forecast and observed values are strongly correlated. In particular, the severe 1958 drought was eminently predictable from this method.

The Sêcas or droughts of Brazil's 'Nordeste', which constitutes the eastern-most corner of the continent (Fig. 1), have recurrently led to starvation and mass exodus, and their eventual prediction has remained a central concern¹. Earlier diagnostic studies^{2–7} have elucidated the general circulation mechanisms of the Sêcas. Drawing on this diagnostic research and combining dynamical

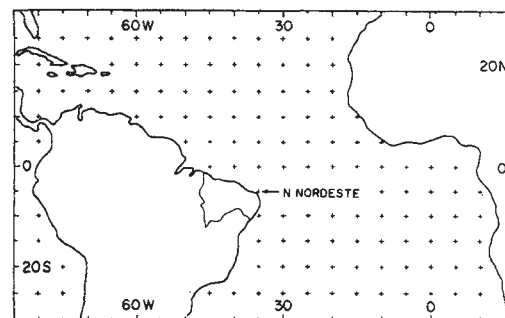


Fig. 1 Orientation map showing the northern Nordeste and 5° square areas for which ship observations were compiled.

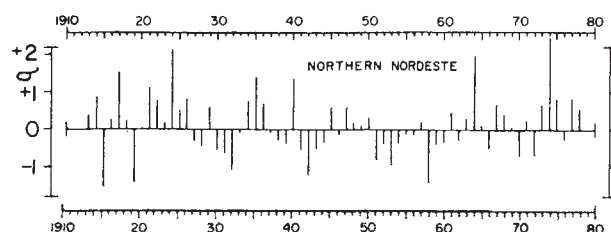


Fig. 2 All-station average of normalized departure of annual rainfall for the northern Nordeste.

and statistical approaches, a method has been developed for the prediction of seasonal rainfall from the antecedent circulation departures. An early report of this work is made here, while full details will be presented elsewhere in due course⁸.

The surface circulation in the tropical Atlantic is documented by ship observations stemming from the TDF-11 deck of the National Climatic Center at Asheville, North Carolina, compiled into 5° square values⁴ (Fig. 1). The record of 1921–72 is used here, and elements of interest include sea level pressure (SLP), zonal (u) and meridional (v) components of resultant surface wind, sea surface temperature (SST), and total cloudiness. Rainfall observations for north-east Brazil are in part published⁹. Also used are selected observation series from other parts of the globe.

The march of the seasons in north-east Brazil is dominated by the latitudinal migration of the near-equatorial trough of low pressure and associated convergence zone over the western North Atlantic, sandwiched as they are between the subtropical highs of either hemisphere. During the Northern Hemisphere winter the trough and convergence zone shift southward, reaching a southernmost position in March and April. This is the peak of the rainy season in the drought-prone northern Nordeste, on which the present study is focused.

A collective of stations in the northern Nordeste (Fig. 1) are combined into rainfall index series of all-station normalized departure³. This procedure safeguards against the notorious space and time variability of tropical rainfall, so that the resulting index series are representative of the hydrometeorological conditions of large homogeneous areas. The October–September index ascribed to the calendar year containing the March–April rainy season peak, is plotted in Fig. 2. Such indices were also compiled for various portions of the hydrological year. Figure 2 illustrates the pronounced interannual variability of rainfall in the northern Nordeste, including the severe drought year 1958, remembered for its particularly serious social and economic consequences^{1,3,7}.

Analyses of large-scale circulation patterns³ show that the Sêcas of the northern Nordeste are characterized by an equatorward expansion of the South Atlantic, and a poleward retraction of the North Atlantic high, associated with a northward displacement of the enclosed near-equatorial trough of low pressure. Concurrently, the zonally oriented bands of maximum cloudiness and precipitation stay farther north, the North Atlantic

Table 1 Percentage of explained variance, period 1921–40 plus 1946–56, for regression on March–April and March–September rainfall index

	O	N	D	J	F	N
Regression on						
March–April	18	55	25	63	56	71
March–September	34	61	48	66	71	—

trades weaken, and the South Atlantic trades become stronger than in the long-term mean. The SST pattern shows positive departures in a broad band across the North Atlantic and anomalously cold waters in most of the South and equatorial Atlantic. The following three factors apparent in the surface circulation seem conducive to drought: (1) the near-equatorial convergence band stays unusually far to the north of the northern Nordeste; (2) the anomalously cold south equatorial waters hamper the moisture content and instability of boundary layer flow upstream from the Nordeste; (3) the perennial contrast between warm north equatorial waters and cold waters to the south of the equator is enhanced. It has been hypothesized⁷ that such a juxtaposition of SST anomalies would induce a meridional circulation cell with subsidence over the Nordeste.

During abundant rainy seasons in the northern Nordeste, departure patterns are approximately inverse to those typical of drought years. Anomaly configurations in the large-scale atmospheric and oceanic fields evolve gradually in the course of the preceding months³.

The aforementioned diagnostic research suggests dynamically plausible predictors of north-east Brazil rainfall anomalies. Thus droughts may be heralded by: anomalously low/high pressure on the equatorward side of the North/South Atlantic subtropical high, enhanced easterly wind component over the low-latitude Atlantic, weakened northerly over the North Atlantic and enhanced southerly wind component over the equatorial and South Atlantic, positive SST departures in a zonally oriented band across the North Atlantic and anomalously cold surface waters in the equatorial South Atlantic, as well as an anomalously far northerly position of the near-equatorial belts of maximum surface convergence and cloudiness. Inasmuch as the pressure distribution over the Atlantic—which is instrumental in establishing the circulation patterns characteristic of the north-east Brazil rain-fall anomalies—appears related to large-scale mass redistributions of the Southern Oscillation type³, various indications of the Southern Oscillation and parameters from more remote parts of the globe are of interest.

While the rainfall index series plotted in Fig. 2 extends from 1913–78, our ship data bank covers 1911–72. However, because of the scarcity of observations in the first decade of this period, only the record since 1921 is used here. With a view towards

identifying suitable predictor candidates, isocorrelate maps were constructed of the October–September north-east Brazil rainfall index with SLP, SST, u and v wind components, and cloudiness over the Atlantic. The highest correlations plausibly occur in the areas described above as being meteorologically significant for the various atmospheric and oceanic fields. The regions of correlation exceeding 0.3 in absolute value were identified and for the combination of adjacent 5° blocks with correlation of same sign and exceeding 0.3, time series were compiled to serve as input to a regression model. This screening is intended to safeguard against noise. Other predictor candidates considered include pre-season rainfall in the northern Nordeste itself, rainfall at Georgetown and Paramaribo in the Guyanas, pressure at Port Stanley in the South Atlantic, temperature in the southeastern United States, India rainfall¹⁰, and Wright's^{11,12} Southern Oscillation index.

Of 17 candidate series, 11 possess a correlation with north-east Brazil rainfall exceeding 0.3 in absolute value, and were input to a stepwise multiple regression scheme with a 5% significance margin^{13,14}. This identified as most important predictors SLP, SST, and u and v wind components over the tropical Atlantic. Of further use was the pre-season rainfall in northern north-east Brazil itself, and the May–June–July Southern Oscillation index value^{11,12}.

Regression models were constructed from the record 1921–40 plus 1946–56, for all months from October through March for two 'predictands', namely the March–April and the March–September rainfall indices. Part A of Fig. 3 is a scatter diagram for January of 'regressed' versus 'observed' values of the March–April rainfall index; the other models are not depicted here. Table 1 lists the variance explained by the regression models for the various months. The variance explained generally increases as one approaches the peak rainy season, no hypothesis being offered for the remarkably weaker relation in December. The peak March–April and March–September rainfalls are specified to a very high degree by the departure ensemble of the general circulation in February and March. However, February leaves no lead time for the practical use of the forecast, while November accounts for a smaller percentage of the rainfall variability. Accordingly, the January models merit particular attention.

The regression models developed from the 1921–40 plus 1946–56 record were used to predict the rainfall anomalies of the 15 years 1958–1972. Part B of Fig. 3 is a scatter diagram of the January model predicting the March–April rainfall index. No details are presented here for the other January model (March–September rainfall) and the two November models. Information such as part A of Fig. 3 serves as a background reference for rainfall index forecasts for years beyond the base period of the regression models, as presented in part B of Fig. 3.

As a basis for the appraisal of forecast performance, various forms of evidence are considered. (1) The scatter diagram in part B of Fig. 3 shows a positive relation between forecast and observed values, although the scatter is larger than for the regression model plotted in part A. (2) Correlation coefficients between forecast and observed values such as given in part B of Fig. 3 are high. (3) In addition, skill scores¹⁵ and stochastic joint probabilities¹⁶ were calculated. These also indicate appreciable forecast skill. Details are not discussed here, but the pertinent information is contained in Fig. 3. In context, the various measures of prediction quality all bear out a remarkable forecast performance. The forecasts are particularly good for the extreme drought year 1958.

A wide range of dynamically plausible predictor candidates was examined, but it is found that the large-scale atmospheric and oceanic fields in the tropical Atlantic are most essential for the prediction of extreme hydrometeorological regions in the northern Nordeste. For operational applications, such parameters are required on a timely basis. Remote sensing by satellite appears an obvious choice, of particular promise being lower-tropospheric cloud motion vectors, SST, and cloudiness or its proxies. It must be realized, however, that time series of inter-

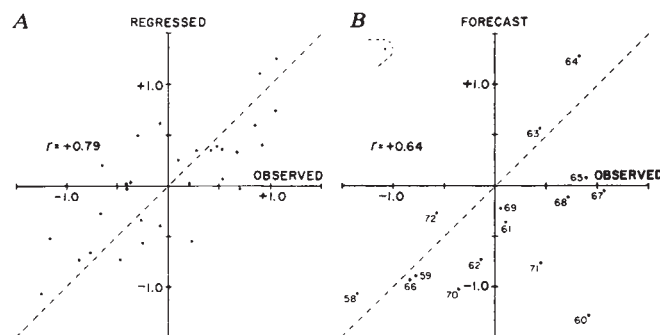


Fig. 3 Scatter diagrams of March/April rainfall index based on observations that would exist by the end of January. A, Regression, period 1921–40 plus 1946–56, correlation coefficient $r = +0.79$. B, Forecast, period 1958–72, with numbers indicating the years, $r = +0.64^{**}$. (Two asterisks denote significance of correlation coefficient at the 1% level.) Broken lines denote 45° angle. Quenouille's¹⁷ method is used to account for the reduction of effective number of degrees of freedom due to persistence.

nally consistent parameters extending over at least ten years are needed, absolute calibration not being essential. Such a satellite-derived reference climatology has not yet been created. The development of an operational monitoring and prediction system entails a substantial material commitment. This is something that deserves to be considered seriously in the light of the demonstrated predictability of northeast Brazil droughts and their social and economic impact.

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Evidence against surface state limitations on efficiency of p-Si/CH₃CN junctions

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We report here the first efficient p-type Si-based semiconductor-liquid junction system. p-Type Si photocathodes have been previously reported to yield open circuit photovoltages, V_{oc} , of 0.38–0.40 V with several redox systems^{1–8}. Photocurrent-voltage studies of p-Si cathodes in CH₃CN solvent have demonstrated 2.5% efficiency for conversion of 632.8-nm light to electricity with the N,N' -dimethyl-4,4'-bipyridinium^{2+/+} redox system¹, and 0.5–2.4% with other macrocyclic complexes^{7,8}. Such modest energy conversion efficiencies for small band gap semiconductors have been attributed to surface states which pin the semiconductor Fermi level^{6–9} and promote recombination processes¹⁰. However, we find that p-type Si/CH₃CN interfaces can yield solar conversion efficiencies in excess of 10%, and can display open circuit photovoltages within 0.08 V of the theoretical limit for an ideal junction.

We have chosen the cobaltocene⁺⁰ (CoCp₂⁺⁰)/1.0 M [(C₂H₅)₄N]⁺[BF₄][−]/CH₃CN redox system to probe the interface behaviour of p-Si in CH₃CN solvent. (CoCp₂)⁺(PF₆)[−] is sufficiently soluble in CH₃CN to be used in concentrations which minimize mass transport polarization losses at the working electrode. In addition, the electrochemically reversible^{11,12} CoCp₂⁺⁰ system possesses an $E^{0'}$ (−0.95 V/SCE) sufficiently negative to effect a large degree of band bending at the semiconductor-liquid interface. Uncompensated ohmic resistance losses can be minimized with proper cell design in this highly conductive

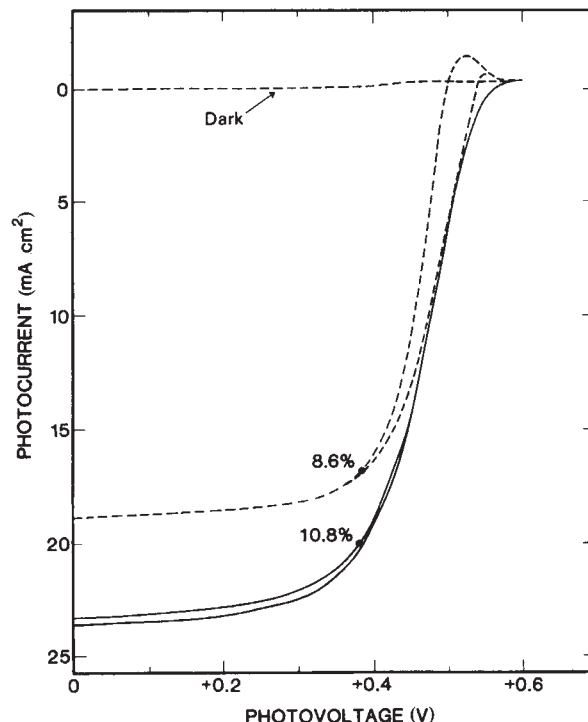


Fig. 1 Current-voltage properties of a single crystal p-type Si photocathode in a stirred CH₃CN/1.0 M [(C₂H₅)₄N]⁺[BF₄][−]/0.2 M cobaltocenium/0.5 mM cobaltocene solution. Light intensity is 70 mW cm^{−2} from an ELH-type tungsten-halogen solar simulator, and photocurrent-voltage curves are uncorrected for any losses due to optical reflection or solution absorption. —, Shiny Si surface obtained from a 10-s etch in 48% aqueous HF, followed by a 10-s rinse with H₂O, then a 10-s rinse with CH₃CN. —, Matte-textured surface¹³ in identical illumination conditions. Both current-voltage curves were measured potentiostatically (50 mV s^{−1}) versus a Pt reference electrode (−0.82 V/SCE) through a Luggin capillary, and represent photoelectrode efficiencies of the semiconductor cathode.

($\rho = 18 \Omega\text{-cm}$) electrolyte; thus, all current-voltage parameters have been measured under potentiostatic control versus a Pt reference electrode in a 0.2-mm outer diameter Luggin capillary. Only when all of the above criteria are met might one conclude that any inefficiencies are due to surface states at the interface.

Use of a mirror-finished p-type Si electrode (HF-etched, 1.5 $\Omega\text{-cm}$ resistivity, (100) oriented) in the optimized 1.0 M [(C₂H₅)₄N]⁺[BF₄][−]/0.2 M CoCp₂⁺/0.5 mM CoCp₂/CH₃CN system with 70 mW cm^{−2} of ELH-type tungsten-halogen irradiation^{13–15} yields V_{oc} of 0.48–0.52 V, short circuit photocurrent densities, J_{sc} , of 20–21 mA cm^{−2}, fill factors of 0.52–0.55, and photoelectrode efficiencies for conversion of incident light to electricity of $8.5 \pm 0.8\%$ (Fig. 1). Texturing of the photoelectrode surface to expose (111) planes and produce a matte black surface yields decreased reflectivity¹³ and produces a system with $V_{oc} = 0.49\text{--}0.53$ V, $J_{sc} = 23\text{--}26$ mA cm^{−2}, and efficiencies of $10.5 \pm 1.0\%$. Actual direct sunlight yields similar behaviour, and at 70 mW cm^{−2} of insolation on matte-etched surfaces, we observe $V_{oc} = 0.49\text{--}0.53$ V, $J_{sc} = 23\text{--}26$ mA cm^{−2}, and efficiencies of $10.5 \pm 1.0\%$.

The photocurrents depicted in Fig. 1 are certainly not due to cathode corrosion. With suitable precautions to exclude air and water from the cell, we have maintained stable ($\pm 10\%$) short circuit currents at AM2 photocurrent densities (> 20 mA cm^{−2}) for passage of $> 2 \times 10^3$ C cm^{−2} through the interface. Longer term stability of this system has not been investigated because of the documented photodecomposition processes of CoCp₂ (ref. 11); however, other soluble, outer-sphere redox couples should be available which would yield similar photocurrent-

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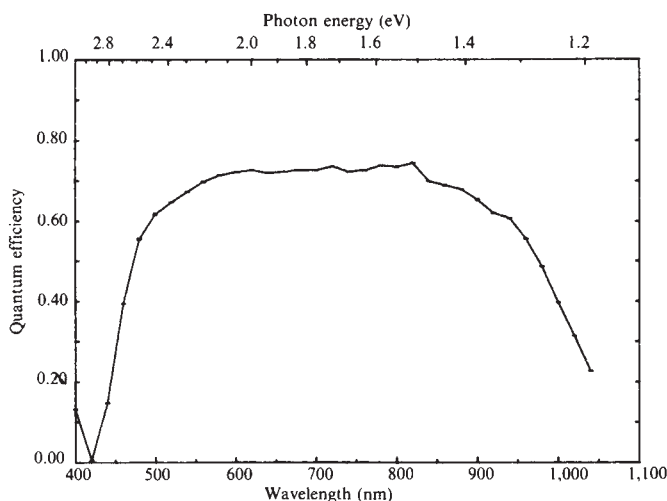


Fig. 2 Spectral response of the short circuit quantum yield plotted against wavelength of incident photons for the p-Si/CH₃CN/cobaltocene^{+/0} system. Average incident light intensity is 1 mW cm⁻², and quantum efficiencies ($\pm 10\%$) are uncorrected for losses due to optical reflection or solution absorption of light. Shiny p-Si cathodes yield 20–30% less current than a matte-textured surface, but both electrodes exhibit excellent response over a broad spectral range.

voltage parameters. Our stability and efficiency measurements represent photoelectrode efficiencies of the working electrode only, and do not represent actual operating two-electrode solar cells. However, the system certainly illustrates that it is possible to observe excellent junction behaviour for p-Si photocathodes in the proper conditions.

The photocurrent response versus incident photon energy for mirror-finished p-Si cathodes is depicted in Fig. 2. The onset of photocurrent is in accord with the 1.1-eV absorption edge of Si, and the high-energy cutoff is attributable to solution absorption of light. An integration of the spectral response properties in Fig. 2 with the solar spectral input yields short circuit photocurrent densities which are in excellent agreement with experimental J_{sc} values. The observation of significant photocurrent near the Si absorption edge implies that unthermalized hot carriers do not provide the sole source of photocurrent flow at these p-Si/CH₃CN junctions. In addition, the high absolute value of the short circuit quantum efficiency, both at low light intensities and solar irradiance levels, yields little evidence for surface recombination sites at the p-Si/CH₃CN interface.

We view these results as consistent with observations in our laboratory^{13,17} and by others^{18,19} that the chemistry of the Si/SiO₂ interface will preclude substantial carrier recombination by surface states. With a minority carrier diffusion length of 300 μ m and a dopant density of 2×10^{15} carriers cm⁻³, photocurrent densities of 20 mA cm⁻² imply a bulk diffusion-limited open circuit voltage of 0.59 V (refs 20–22). Thus, the V_{oc} of 0.48–0.52 V at 20–21 mA cm⁻² for the p-Si/CH₃CN system is within 0.08 V of the maximum attainable value for any junction fabricated with our semiconductor samples. Furthermore, the V_{oc} of p-Si/CH₃CN junctions substantially exceed V_{oc} values typically found for direct p-Si/Au or p-Si/Pt Schottky barriers ($V_{oc} < 0.20$ – 0.25 V)²³. In fact, the V_{oc} values at high light intensity (> 1 W cm⁻²) for the p-Si/CH₃CN system (> 0.55 V) exceed reported values of barrier heights for p-Si Schottky barriers with metals such as Ag, Au, Cr, Cu, Mo, Ni, Pb, Pd, Pt and W (ref. 20). Clearly, intrinsic surface states do not dominate the chemistry at both the semiconductor/liquid and semiconductor/metal interfaces.

Our data on the p-Si/CH₃CN interface yield no evidence for surface state limitations which would prevent the rational design and study of efficient semiconductor–liquid junctions. Investigations of the p-Si system are thus consistent with recent studies

of other common, small band gap semiconductors (n-Si (ref. 13), n-GaAs (ref. 24), n-GaAs_{1-x}P_x (ref. 25)) which suggest that control of bulk electrode properties and of solution overpotential losses can eliminate most inefficiencies previously ascribed to surface states at the semiconductor–liquid interface. Extension of the CoCp₂^{+/0} redox system, and similar metallocenes, to other photocathode materials is underway.

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Photochemical dehydrogenation of ethanol in dilute aqueous solution

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The need for alternative sources of energy has stimulated research into the storage of sunlight as chemical potential. Many systems have been investigated but photogeneration of H₂ seems to be the most practical. The cyclic photodissociation of water has yet to be realized in homogeneous solution using visible light, although heterogeneous photosystems are known^{1–3}. Efficient photogeneration of H₂ in homogeneous conditions can be achieved^{4–7} at the expense of an added electron donor but such systems have little application in practical devices. Replacement of the sacrificial donor with waste material, for example sulphide⁸, offers a route for improving these systems and, here we describe efficient H₂ photogeneration from a system using aqueous ethanol, as available from low grade fermentation, as donor. To couple the photoproduction of H₂ to the oxidation of ethanol we have used NADH/alcohol dehydrogenase as a relay.

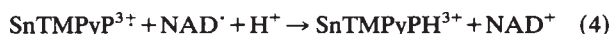
Dichloro tin(IV) *meso*-tetrakis (*N*-methyl-4-pyridyl)porphyrine (SnTMPyP⁴⁺) dissolves readily in water throughout the entire pH range and such solutions absorb strongly all light

below 620 nm and are therefore capable of collecting some 40% of the energy available in sunlight. Upon excitation with visible light ($\lambda < 620$ nm), the triplet excited state of the metalloporphyrin is formed in high yield ($\phi_T \sim 0.95$) and it is quite long-lived ($\tau_T = 0.9$ ms) in the absence of oxygen. The triplet state is a powerful oxidizing agent and is quenched by NADH at the diffusion controlled bimolecular rate limit due to transfer of a single electron, as shown by flash photolysis studies.



$$pK = -4$$

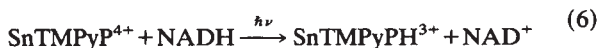
The one-electron reduction product of the metalloporphyrin is the π -radical anion (SnTMPyP^{3+}) which can be identified by its characteristic absorption spectrum⁹. The π -radical anion is unstable with respect to disproportionation at $pH < 12$, although it persists for many minutes in alkaline solution, and it is also reduced rapidly by the oxidized form of the donor.



Both processes result in formation of the metallophlorin (SnTMPyPH^{3+}), which is a protonated π -dianion and has a characteristic absorption maximum centred at 830 nm (refs 10, 11). The phlorin is stable in outgassed aqueous solution over the pH range 4–14, although it undergoes acid/base equilibration and is quantitatively reoxidized to the original metalloporphyrin on aeration. For light of $\lambda 550$ nm, the quantum yield for formation of the phlorin was 0.60 ± 0.05 for concentrations of NADH of 2×10^{-3} mol dm⁻³ and SnTMPyP^{4+} of 1×10^{-5} mol dm⁻³ at pH 7.5. Since the oxidized form of the donor can also reduce ground state metalloporphyrin



the overall process requires one photon to drive a two-electron reduction of the porphyrin



It should be emphasized that the porphyrin/phlorin cycle is exceptionally clean for SnTMPyP^{4+} at $pH > 4$ and, unlike most metalloporphyrins studied to date^{9,10}, irreversible formation of the chlorin is unimportant.

When colloidal Pt (ref. 12) is present, irradiation ($\lambda < 620$ nm) of the solution results in formation of H_2 and the phlorin is no longer observed as a reaction product. Depending on pH , the phlorin, the π -radical anion and NAD^+ are all capable of reducing protons to H_2 on the surface of a Pt catalyst. With a Pt concentration of 10^{-4} mol dm⁻³ and irradiation with $\lambda 550 \pm 10$ nm, the quantum yield for formation of H_2 was found to be 0.43 ± 0.05 at pH 7.5. Thus, conversion of the reducing equivalents into H_2 is almost quantitative and H_2 formation is not restricted to neutral solution but occurs throughout the range $5 < pH < 10$. It was found that Pt catalysed formation of the metallochlorin, albeit ineffectively, so that at high concentrations of Pt the cycle with respect to the porphyrin was no longer entirely clean. Although very efficient, the above cycle is sacrificial in that the evolved H_2 is at the expense of oxidation of NADH to NAD^+ and there is a concomitant increase in pH .



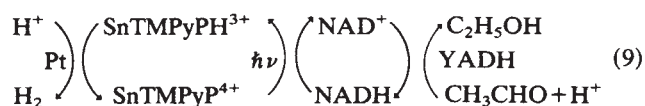
In our conditions, there is no experimental evidence that Pt catalyses reduction of NAD^+ by H_2 although this reaction is expected to occur on standing in the dark.

Regeneration of NADH from NAD^+ can be achieved readily using ethanol and an enzyme catalyst such as yeast alcohol dehydrogenase (YADH).



Thus, irradiation of SnTMPyP^{4+} (10^{-5} mol dm⁻³) with green light in N_2 -purged aqueous solution at pH 7.5 containing NADH (2×10^{-3} mol dm⁻³), colloidal Pt (10^{-4} mol dm⁻³), YADH (25 mg dm⁻³) and ethanol (2% v/v) resulted in sustained generation of H_2 . In a typical experiment, the rate of H_2 formation was approximately 440 cm³ per dm³ of solution per h at an incident light intensity of about 10^{20} photons min⁻¹. On prolonged irradiation, turnover numbers of 86 and 131,000 respectively have been observed for NADH and YADH, although that for NADH is not exhaustive. Only a small fraction of the metalloporphyrin is lost (via chlorin formation) in these conditions whilst the ethanol can be converted quantitatively into acetaldehyde and H_2 and the quantum yield for formation of H_2 remained at 0.43 ± 0.05 . The solution pH remains constant during the overall reaction and neither acetaldehyde nor its solvate inhibit H_2 evolution. The rate of H_2 formation shows no significant decrease until the YADH or ethanol has been consumed. The rate and quantum yield of H_2 formation compare favourably with any previously reported sacrificial H_2 evolving photosystem while the longevity of the present system greatly exceeds that of any previous system.

Overall, the photoreaction may be summarized by the following scheme:



Dehydrogenation of ethanol



$$\Delta G^0 = 42 \text{ kJ mol}^{-1}$$

is thermodynamically uphill so that energy is stored during the reaction. The overall efficiency for storage of light energy ($\lambda = 620$ nm) is $(0.43 \times 42/193)$ 9.4%. The photoreaction might offer a useful alternative to distillation of ethanol from low grade fermentation processes. However, the idea of using an enzyme to regenerate the electron donor is not restricted to ethanol but D-glucose or lactate could be used instead. Finally, we note that the phlorin is extremely stable in the absence of air so that the photoreaction could be used in a flow cell enabling the H_2 to be liberated at a specific site.

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Large mantle heterogeneity beneath French Polynesia

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New strontium, lead and neodymium isotope compositions of volcanics from two volcanic island chains of French Polynesia (Marquesas, Australes) are presented. The data from Marquesas fall in the well-established oceanic islands field and are very similar to those of the Society Islands of French Polynesia. In contrast, Tubuaii Island basalts from the Australes chain exhibit very low $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (~ 0.7028) and the highest $^{206}\text{Pb}/^{204}\text{Pb}$ (~ 21.1) and $^{208}\text{Pb}/^{204}\text{Pb}$ (~ 40.4) ratios ever reported for an oceanic island. Their ϵ_{Nd} value is $\sim +4.9$. These data are similar to those of St Helena, although even more extreme. This convergence indicates that a comparable and as yet undefined process took place in the mantle beneath the Atlantic and Pacific oceans. The isotopic data for the Polynesian volcanics encompass almost the entire range of Sr and Nd isotopic ratios found in oceanic islands ($^{87}\text{Sr}/^{86}\text{Sr} = 0.7028\text{--}0.7083$; $^{143}\text{Nd}/^{144}\text{Nd} = 0.51257\text{--}0.51297$) and more than half of the Pb isotope range ($^{206}\text{Pb}/^{204}\text{Pb} = 18.9\text{--}21.1$; $^{208}\text{Pb}/^{204}\text{Pb} = 38.9\text{--}40.4$).

Mid-ocean ridge basalt (MORB) and oceanic island volcanism are known to originate from totally distinct mantle sources and to have been separated for at least 1,000 Myr. Despite the number of models¹⁻⁹, mostly based on isotopic data, the following points are still matters of discussion: (1) What is the depth of the ocean island basalts source regions? Are they located in the upper or in the lower mantle? (2) Are the large variations in isotopic ratios observed in volcanic rocks at the present time the result of primary heterogeneity of the U/Pb, Rb/Sr and Sm/Nd ratios at the time of the formation of the mantle or of later processes? (3) In the case of the lower mantle hypothesis, what is the extent of the interaction with the core? (4) Does altered and subducted oceanic crust or continental material contribute to the oceanic island isotopic pattern? (5) To what degree have oceanic island magmas been chemically and isotopically contaminated as they rise through the depleted upper mantle?

Although a number of oceanic islands are now well documented regarding isotopes there are only a few high-precision data on Pb, Sr and Nd isotopes on the same samples. In addition, a strong emphasis has been put on Atlantic islands and few data are available on the Indian and Pacific oceans. A more precise answer to these questions will be given when the whole picture of isotopic pattern (Pb, Sr, Nd, Hf, rare gases) on all oceans becomes available.

We report here some new measurements on French Polynesia which due to its large surface area (2,200,000 km²; 8–18 lat. south and 135–155 long. west) allows us to study the mantle heterogeneity on a larger scale than was possible with the other oceanic islands.

In the central Pacific Ocean, four volcanic chains are aligned parallel to the Hawaiian chain: the Marquesas Islands, the Gambier–Tuamoutou Islands, the Society Islands and the Australes Islands (formally called Tubuaii Islands) (Fig. 1). Each of these chains can be interpreted as being the result of a hot spot¹⁰ which is located at the south-east end of each chain.

In the Marquesas Islands, volcanic activity ranges in age from 6.3 ± 0.2 Myr (in Eiao Island) to 1.30 ± 0.02 Myr (in Fatu Hiva Island)^{11,12}. In the Australes Islands, volcanism extends from 18 Myr (at the north-west end of the chain¹³⁻¹⁵) to the present as indicated by active volcanism on MacDonald seamount at the south-east end of the chain^{16,17}.

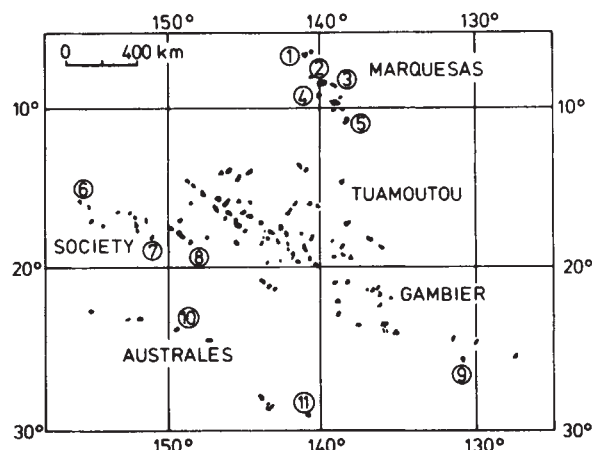


Fig. 1 Location of the Polynesian islands in the Central Pacific. Numbers refer to the islands: 1, Eiao; 2, Nuku Hiva; 3, Ua Huka; 4, Ua Pou; 5, Fatu Hiva; 6, Maupiti; 7, Tahiti; 8, Mehetia; 9, Pitcairn; 10, Tubuaii; 11, MacDonald. Ranges in lava compositions are as follows: Ua Pou—a potassic series, picrite to K-phonolite²¹; Ua Huka—undifferentiated basalt²⁰; Nuku Hiva—alkali-olivine basalt to alkali-rich quartz-trachyte²²; Tubuaii—five distinct series are observed²³: (i) basal series of the main volcano (12–10.5 Myr) composed of undifferentiated transitional basalts (5436); (ii) medium series of the main volcano (9.5–6.8 Myr) composed of undifferentiated alkali basalts (5434); (iii) top series of the main volcano ranging in composition from differentiated basanites (5433) to phonolites; (iv) a group of later intrusions (≈ 5.7 Myr) composed of analcimites (5435); (v) the succession of the secondary volcano located on the west part of the island (10.5–8 Myr) ranging in composition from murites (5437) to phonolites. As noted above, one sample has been selected in each series, each of these samples representing the more primitive liquid of the series.

In both the Marquesas and Australes Islands, the volcanic products are of alkalic affinity. Each island is formed by the succession of at least two different volcanic sequences¹⁸, the alkalic character increasing from the beginning to the end of the sequence¹⁹.

We analysed samples collected in three different islands of the Marquesas chain: Ua Pou, Ua Huka and Nuku Hiva, as well as samples from Tubuaii Island in the Australes chain, and the results are presented in Table 1 and in the legend to Fig. 1.

Lead chemistry and mass spectrometry have been performed in Rennes using the method described by Vidal and Clauer²⁴. Part of the Sr data was obtained in Rennes using the technique described by Chauvel²⁵. The other part of the Sr data as well as the Nd data were obtained in Mainz using the analytical procedures reported by White and Patchett²⁶.

Sr, Pb and Nd isotopic ratios are reported in Table 1. Due to their low ages (2–10 Myr) and their low Rb/Sr ratios, the *in situ* correction for the increase of $^{87}\text{Sr}/^{86}\text{Sr}$ by radioactive decay is for most of the samples within the analytical uncertainties. However, for a few samples the correction is on the fourth decimal place. The measured Nd isotopic ratios can be considered as initial ratios because the rocks are very young. For Pb isotopes no correction has been made due to the lack of data regarding Th and U contents. Only $^{206}\text{Pb}/^{204}\text{Pb}$ ratios are actually sensitive to this correction, although they are also generally considered as initial ratios. In fact, in the present study, the very close grouping of $^{206}\text{Pb}/^{204}\text{Pb}$ ratios within one single island whatever the petrographic type enables us to consider the observed ratios as closely approaching the initial values.

The results are plotted on four diagrams: $^{207}\text{Pb}/^{204}\text{Pb}$ against $^{206}\text{Pb}/^{204}\text{Pb}$ and $^{208}\text{Pb}/^{204}\text{Pb}$ against $^{206}\text{Pb}/^{204}\text{Pb}$ (Fig. 2a, b), $^{143}\text{Nd}/^{144}\text{Nd}$ against $^{87}\text{Sr}/^{86}\text{Sr}$ (c) and $^{87}\text{Sr}/^{86}\text{Sr}$ against $^{206}\text{Pb}/^{204}\text{Pb}$ (d).

In all diagrams, the data from Marquesas plot in the middle of the field defined by the other oceanic islands. They are particularly comparable to the data of Society Islands (refs 27,

Table 1 Isotopic results for the Polynesian samples

Sample	Type or rock	Rb	Sr	Pb	$^{87}\text{Sr}/^{86}\text{Sr}$ (measured)	$^{87}\text{Sr}/^{86}\text{Sr}$ (initial)	$^{206}\text{Pb}/^{204}\text{Pb}^*$	$^{207}\text{Pb}/^{204}\text{Pb}^*$	$^{208}\text{Pb}/^{204}\text{Pb}^*$	$^{143}\text{Nd}/^{144}\text{Nd}$	ϵ_{Nd}
Marquesas											
Ua Pou											
5424 (73F)	K-Na Basalt	42	531	3.3	$\pm 0.705131 \pm 21$	0.70512	19.23	15.62	39.23	0.512710 ± 14	+1.4
5425 (74P)	K-phonolite	212	418	18	$\pm 0.705419 \pm 78$	0.70536	—	—	—	—	—
5426 (60Y)	K-Na phonolite	229	126	20	$\pm 0.704866 \pm 86$	0.70466	19.24	15.63	39.26	—	—
5427 (73R)	K-benmoreite	117	1,032	10	$\pm 0.704743 \pm 16$	0.70473	19.22	15.63	39.12	0.512750 ± 11	+2.2
5428 (74h)	K-basalt	106	1,300	5.4	$\pm 0.705225 \pm 72$	0.70522	—	—	—	—	—
Ua Huka											
5429 (67H)	Basanite	12	617	2.6	$\pm 0.704584 \pm 54$	—	18.94	15.58	38.92	—	—
5430 (68F ₂)	Basalt	43	450	4.5	$\pm 0.705614 \pm 22$	—	19.15	15.65	39.29	0.512741 ± 34	+2.0
5430 (68F ₂)	Leached sample	—	—	—	$\pm 0.705688 \pm 25$	—	—	—	—	—	—
Nuku Hiva											
5438 (70X)	K-trachyte	147	365	9.7	$\pm 0.704487 \pm 17$	0.70443	19.02	15.61	38.98	0.512765 ± 12	+2.5
5439 (78A)	Basalt	21	415	11	$\pm 0.703792 \pm 30$	0.70378	19.11	15.57	38.88	0.512889 ± 28	+4.9
5440 (77t)	Basalt	25	538	2.8	$\pm 0.704231 \pm 68$	0.70422	19.08	15.58	39.01	—	—
5442 (10E)	Basalt	7	412	1.8	$\pm 0.703040 \pm 21$	0.70304	19.13	15.58	39.13	0.512971 ± 17	+6.5
5443 (76Z)	Na-trachyte	137	635	8.4	$\pm 0.704361 \pm 72$	0.70433	19.05	15.62	39.16	—	—
5444 (76F)	Benmoreite	174	457	—	$\pm 0.705134 \pm 64$	0.70507	—	—	—	—	—
Australes											
Tubuaii											
5433 (TU 23)	Basalt	27	831	2.8	$\pm 0.702800 \pm 12$	0.70279	21.14	15.76	40.29	0.512886 ± 16	+4.8
5434 (TU 21)	Basalt	20	509	2.5	$\pm 0.702779 \pm 20$	0.70276	21.07	15.76	40.33	0.512882 ± 14	+4.8
5435 (TU 31)	Basalt	51	1,028	8.6	$\pm 0.702793 \pm 21$	0.70277	21.14	15.78	40.44	0.512887 ± 19	+4.9
5436 (TU 29)	Basalt	16	562	1.0	$\pm 0.702793 \pm 18$	0.70278	21.06	15.78	40.30	0.512884 ± 11	+4.8
5437 (TU 4)	Basalt	59	1,301	4.4	$\pm 0.702788 \pm 21$	0.70277	21.11	15.77	40.41	0.512895 ± 17	+5.0

$^{87}\text{Sr}/^{86}\text{Sr}$ ratios are normalized to $^{86}\text{Sr}/^{88}\text{Sr} = 0.1194$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios to $^{144}\text{Nd}/^{146}\text{Nd} = 0.7219$. Values for E & A standard and La Jolla standard measured at the Max-Planck-Institute are $^{87}\text{Sr}/^{86}\text{Sr} = 0.707998 \pm 40$ (2 σ pop) and $^{143}\text{Nd}/^{144}\text{Nd} = 0.511847 \pm 21$ (2 σ pop) respectively. Value in Rennes for NBS 987 standard is $^{87}\text{Sr}/^{86}\text{Sr} = 0.71020 \pm 4$ (2 σ). Concentrations in Rb, Sr and Pb have been measured by the XRF method. Precision is 2% for Rb and Sr contents and 10% for Pb content.

* As estimated from replicates of NBS 981 analysis, maximum errors on $^{206}\text{Pb}/^{204}\text{Pb}$, $^{207}\text{Pb}/^{204}\text{Pb}$ and $^{208}\text{Pb}/^{204}\text{Pb}$ ratios are 0.15% and on $^{207}\text{Pb}/^{206}\text{Pb}$, 0.07%.

† Sr data obtained in Max-Planck-Institute, Mainz.

28 and W. M. White, unpublished data). This suggests that the Marquesas and the Society Islands may originate from similar sources. In contrast, Tubuaii Island field is very different from the other oceanic islands, with the exception of St Helena.

The strontium data for the Marquesas Islands, presented here, are in excellent agreement with those of Duncan and Compston²⁹. Within one single island the spread in $^{87}\text{Sr}/^{86}\text{Sr}$ ratios can be very large. In contrast, the lead isotopic ratios are very homogeneous for each island. The spread in strontium isotopic ratios might be related to some interaction with seawater (which has an $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7091) at the level of the magma chamber. However, in this case, a correlation between $^{87}\text{Sr}/^{86}\text{Sr}$ ratio and Sr content should be observed, which is obviously not the case (Table 1). In addition, Nd isotopes correlate negatively with Sr isotopes (Fig. 2c) and lie on the mantle array. As seawater contamination affects only Sr isotopes and in consequence shifts the points to the right of the mantle array, the present results can be considered indicative of large strontium and neodymium mantle heterogeneity beneath these islands. These data also have a bearing on the genesis of the volcanism within each of the islands: on each, the rocks have isotopic compositions indicating that they cannot have originated from a single primary basaltic melt by a fractional crystallization; either they originate from distinct mantle sources separated for more than 1,000 Myr or they result from the mixing in different proportions of a MORB-type source and of an enriched source. In either case, fractional crystallization occurring in the magma chambers may modify elemental concentrations.

Although each belongs to a separate magmatic suite, the five analysed samples from Tubuaii have remarkably similar Sr, Pb and Nd isotopic compositions. The $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of 0.70278 (a value comparable to those of Ascension, St Helena and Galapagos^{27,30-32}) are among the lowest reported for an oceanic island. In contrast, the $^{206}\text{Pb}/^{204}\text{Pb}$ of 21.10 and the $^{208}\text{Pb}/^{204}\text{Pb}$ of 40.35 are the highest reported for an oceanic island. Of published data, St Helena has the most similar composition, with $^{206}\text{Pb}/^{204}\text{Pb}$ ratios ranging between 20.70 and 20.91 and $^{208}\text{Pb}/^{204}\text{Pb}$ from 40.00 to 40.16³³.

Both in Nd/Sr and Sr/Pb diagrams (Fig. 2c, d) the Tubuaii data plot very close to the St Helena data. This shows that the

St Helena mantle source is not an exception and that beneath both the South Atlantic and South Pacific oceans, the same kind of peculiar mantle region exists. Zindler *et al.*³⁴ propose that the St Helena data indicate a mixture of depleted mantle and recycled oceanic crust, the latter having had a low Rb/Sr ratio, relatively high Sm/Nd and high U/Pb ratio for a long period of time. Low Rb/Sr and $^{87}\text{Sr}/^{86}\text{Sr}$ for the recycled oceanic crust seem unlikely since Hart and Staudigel³⁵ consider that alteration increases both the Rb content and the Sr isotopic composition of the oceanic crust. However, Michard *et al.*³⁶ present data on the East Pacific Rise black smokers which shows that the hydrothermal fluid circulation within the oceanic crust may drastically reduce the Rb content of the oceanic crust and increase its U content. If the effects of the hydrothermal alteration are large enough to counteract the increase of the Rb/Sr ratio due to the addition of sediments and to the superficial seawater alteration, then the Rb/Sr ratio of the altered oceanic crust could decrease to below its original value.

If such a decrease of the global Rb/Sr ratio of the altered crust is realistic, data such as those obtained in St Helena and Tubuaii can be explained by melting of a recycled oceanic crust. An alternative explanation for having a mantle source with long time-integrated low Rb/Sr ratio as well as high U/Pb ratio, has already been proposed by Vidal and Dosso³. These authors proposed that part of the mantle has been depleted in Pb and Rb by extraction of those elements as sulphide phases from the lower mantle to the core. This could be the end-member close to the St Helena and Tubuaii data in the mantle plane.

The data now available for Marquesas and Tubuaii have two general implications regarding the mantle evolution. First, the particular mantle process which produced the St Helena source and which is still not well understood also took place beneath a portion of the Pacific Ocean, and second, the mantle is extremely heterogeneous beneath French Polynesia. Samples from a single island (Nuku Hiva) cover half the observed Sr-Nd isotopic variation in the oceanic islands, and in the three Marquesas islands studied here, almost the entire range is observed. On the scale of the whole of French Polynesia, more than half of the Pb isotope field is observed. These observations suggest that the large number of Polynesian islands scattered over a very large area offer a unique opportunity to study the scales

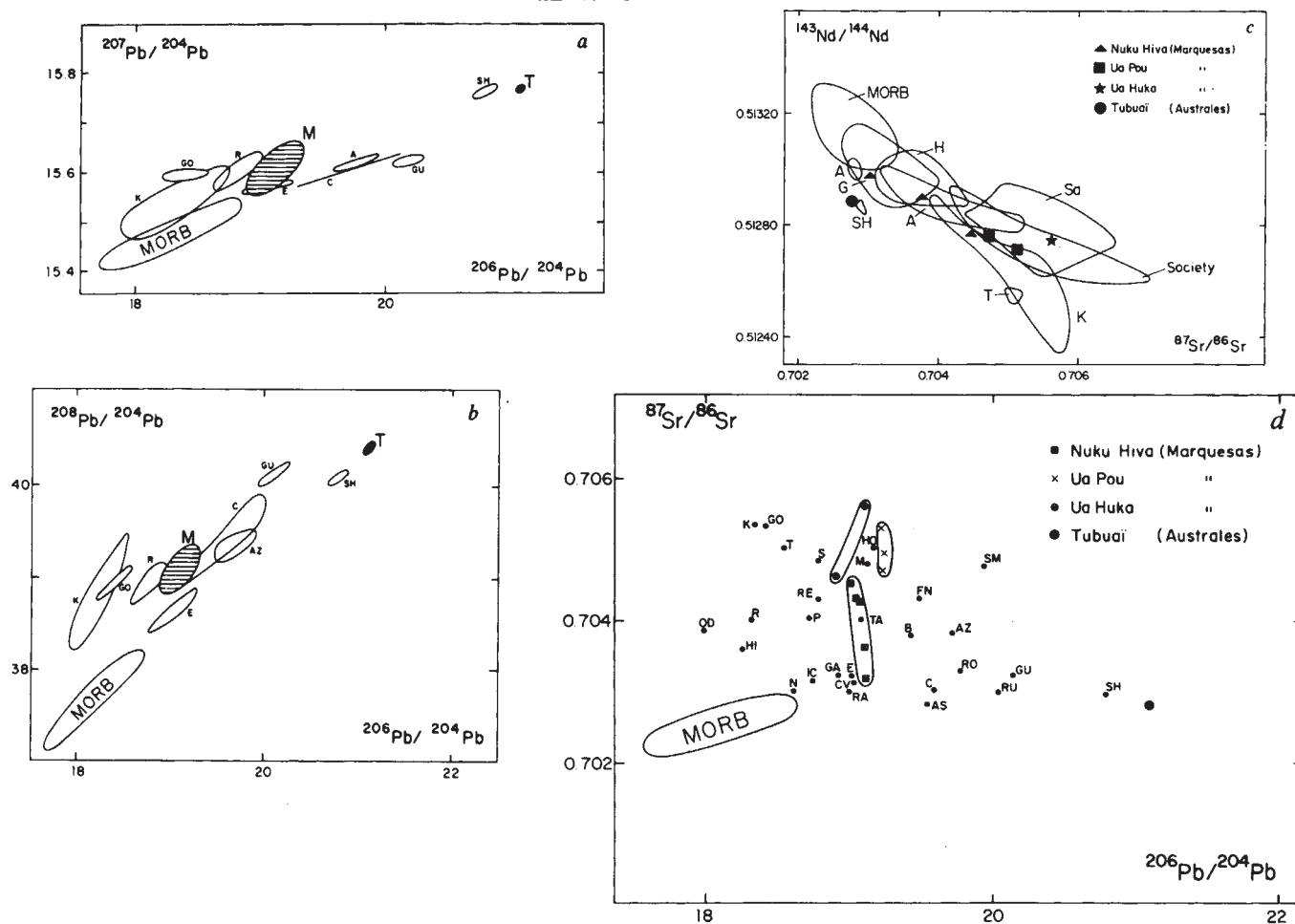


Fig. 2 a, $^{207}\text{Pb}/^{204}\text{Pb}$ versus $^{206}\text{Pb}/^{204}\text{Pb}$ diagram; b, $^{208}\text{Pb}/^{204}\text{Pb}$ versus $^{206}\text{Pb}/^{204}\text{Pb}$ diagram. T, Tubuaii (this work); M, Marquesas (this work); other data are from the literature. SH, St Helena; GU, Guadalupe; AZ, Azores; C, Canary; E, Easter; R, Reunion; GO, Gough; K, Kerguelen. c, $^{143}\text{Nd}/^{144}\text{Nd}$ versus $^{87}\text{Sr}/^{86}\text{Sr}$ diagram. Data from this work are represented by symbols; other data^{27,32,37-42} are only shown by fields. d, $^{87}\text{Sr}/^{86}\text{Sr}$ versus $^{206}\text{Pb}/^{204}\text{Pb}$ diagram. In order to simplify the figure, an average of all data available for each island has been done as already suggested by Zindler *et al.*³⁴. Data are from the compilation made by Sun³³ with the exception of Fernando de Noronha (F.N.), which are unpublished data of Ph.V. and G. Cornen. Abbreviations as follows: K, Kerguelen; GO, Gough; T, Tristan da Cunha; S, Samoa; HO, Hiva Oa; M, Moorea; SM, Sao Miguel; RE, Reunion; P, Penhu; TA, Tahiti; B, Bouvet; AZ, Azores; GA, Galapagos; SH, St Helena; GU, Guadalupe; RU, Rurutu (Australes); RO, Ross; C, Canary; AS, Ascension; RA, Rapa (Australes); CV, Cape Verde; E, Easter; N, Nunivak; IC, Iceland; OD, Oki Dogo; R, Rarotonga; HI, Hawaii.

of mantle heterogeneities. In particular it is striking that each of the three Marquesas islands studied has a unique Pb composition, whereas Sr and Nd vary greatly.

We conclude from our observation first that the volume of the mantle involved in the generation of the lavas observed in one island might, in the case of an idealized cubic volume, have a side smaller than a few tens of kilometres, and second that large Sr and Nd heterogeneities might have persisted on this rather restricted scale over thousands of millions of years. In fact, the actual heterogeneities might be smaller, since the spread defined by the data in the Sr against Nd diagram can be due to a mixing between two sources: the first being time-integrated enriched in incompatible elements, and the second being a MORB-type source.

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Metabolic activity and bioluminescence of oceanic faecal pellets and sediment trap particles

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Zooplankton faecal pellets are important in the vertical transport and exchange of elements in the oceanic ecosystem^{1,2}. Although microbial, and in particular bacterial, processes have been implicated in the decomposition of aquatic faecal pellets³⁻⁷, few quantitative data exist on the metabolic activities and growth characteristics of the associated microorganisms. As part of the Vertex (Vertical Transport and Exchange) programme, we studied these aspects using field-collected faecal pellets of known source and age. Our results, reported here, show that microbial biomass and metabolic activity are highest at the time of egestion, and decline with subsequent incubation. An independent observation from these experiments was the detection of bioluminescence in freshly excreted faecal pellets and in materials collected from sediment traps. These field data support an existing model for the ecological role of bacterial light emission in the mesopelagic zone of the ocean.

In the present study, freshly produced copepod faecal pellets were incubated in sterile filtered seawater in temperature-controlled conditions. Changes in ATP biomass, RNA and DNA synthesis rates (as measures of total microbial production and cell division) and bioluminescence were monitored with time. The data (Fig. 1) show that rates of RNA and DNA synthesis were highest at the time of egestion and decreased with subsequent ageing. Since nucleic acid synthesis rates are positively correlated with cellular biosynthesis⁸⁻¹⁰, it can be concluded that both growth and cell division decreased with time. After 48 h, the RNA synthesis rate was 22% of the initial rate and the DNA synthesis rate had decreased to less than 3% of the original value. Two additional experiments, performed over 96 h, confirmed that microbial rate processes decreased with faecal pellet age. Total microbial biomass (as determined from faecal pellet ATP concentrations) was more variable among experiments. However, in no case was there evidence for an overall increase in the total microbial population during the incubation periods (up to 120 h). ATP values for fresh copepod faecal pellets in these experiments ranged from 70 to 1,156 ng per mg dry weight.

Although low temperatures have been related to decreases in metabolic activity and rates of faecal pellet degradation^{5,11}, there was no apparent response to temperature changes in our experiments. Pellets, aged at a constant temperature of 19°C, showed reductions in rates of RNA and DNA synthesis similar to those of faecal pellets aged as described in Fig. 1 legend.

The decreases we observed in the above experiments were unexpected as the present scenario for faecal pellet breakdown requires that the associated microbial populations exhibit rapid growth and division. Indeed, Honjo and Roman⁴ observed that degradation of the surface membrane of laboratory-produced copepod pellets began within 3 h and was complete after 24 h. The implication that faecal pellets are colonized and degraded by external microbial populations is derived from time-course electron microscopic studies of pellet surfaces³⁻⁵. In our study, however, the high biomass, metabolic activity and luminescence (see below) associated with field-collected faecal pellets at the time of egestion indicated the presence and importance of gut-derived microflora. Furthermore, we were unable to detect any

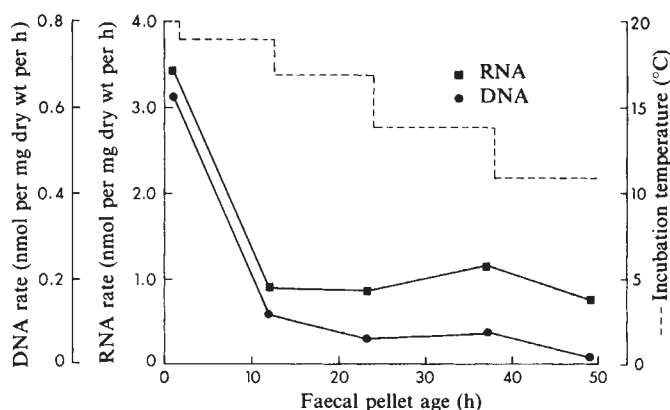


Fig. 1 Changes in rates of RNA and DNA synthesis as a function of faecal pellet age. Fresh faecal pellets from net-collected copepods were obtained by the method of La Rosa²⁹ as modified by Small *et al.*¹³. Known volumes of pellets, retained on a 68 µm Nitex net, were rinsed with distilled water, dried and weighed using a Cahn electrobalance. Faecal pellet material was also diluted 20-fold with 64 µm Nitex-filtered seawater and replicate 1-ml and 0.1-ml aliquots were incubated in small glass vials. The glass vials were moved through a temperature gradient with time (20–11 °C) to simulate the *in situ* conditions that sinking pellets would encounter. The rate at which the vials were rotated was determined by combining pellet sinking rate data¹³ with the temperature regime of the water column. At selected times during the experiment, a 1-ml vial was incubated with 20 µCi ml⁻¹ (final concentration) of [2-³H]adenine (15.5 Ci mmol⁻¹; NEN) for 1 h, after which determinations of rates of RNA and DNA synthesis^{30,31} and ATP³² were made. Vials containing the 0.1-ml aliquots were filtered onto a combusted glass fibre filter and analysed for organic carbon, using a Perkin-Elmer CN analyser, throughout the incubation period. Faecal pellets were also aged in 0.22-µm Millipore-filtered seawater to assess contamination from planktonic bacteria. No differences in nucleic acid synthesis rates or ATP concentrations were observed between pellets incubated in 68 µm and in 0.22 µm-filtered seawater.

major differences between faecal pellets incubated in sterile filtered seawater and in unfiltered seawater. Recent transmission electron microscopic observations of thin sections from copepod faecal pellet interiors⁷ also suggest that internal microbial communities have a major role in faecal pellet breakdown.

Oceanic zooplankton, grown in conditions of food limitation, are known to have higher assimilation efficiencies than coastal plankton or laboratory cultures maintained with high food concentrations¹². Major differences between faecal pellets produced by the two groups have been noted¹³. Microbiological activity may similarly depend on the quality, compaction and composition of the egested pellets. Carbon analyses of our source-term pellets revealed that between 12 and 27% of the faecal pellet mass was organic carbon, which is in agreement with published data^{4,5,14}. However, 50–100% of this organic carbon was living carbon as estimated from our ATP data (using an average C:ATP ratio of 250 (refs 15, 16)). It seems that reduced carbon sources may be insufficient to support the active growth and division of chemoheterotrophic microbial populations and that these organisms substantially reduce their metabolic activity soon after the pellets are voided. This is consistent with the previous observation that faecal pellets produced by euphausiids feeding on natural food sources can remain intact for weeks at temperatures as high as 18 °C (ref. 17).

In vivo bioluminescence, detected with a shipboard photometer, occurred in 70% of all samples surveyed including sediment trap-collected particulate matter, freshly collected zooplankton faecal pellets, crustacean molts, live animals (such as euphausiids, copepods, amphipods, etc.), and particulate detritus collected with plankton nets and water samplers. Long-term kinetics (s to min) revealed two distinct patterns of light emission, possibly reflecting various sources of luminescence. Erratic, luminous flashes predominated in the live animal and

Table 1 Vertical distribution of bioluminescence and ATP from particle interceptor traps (VERTEX II)

Depth (m)	Total sediment trap bioluminescence (10^5 photons $\text{cm}^{-2} \text{s}^{-1}$)	Total sediment-trap ATP (μg)	Specific bioluminescence (photons $\text{cm}^{-2} \text{s}^{-1}$ per pg ATP)
30	17.2	1.30 (0.296)	1.32
120	3.39 (0.024)	2.09 (0.172)	0.167
200	2.96 (0.041)	2.52 (0.235)	0.117
400	1.21 (0.022)	1.04 (0.094)	0.116
700	1.30 (0.023)	0.742 (0.161)	0.175
800	1.11 (0.009)	0.833 (0.067)	0.133
900	1.36 (0.020)	0.083 (0.025)	1.64
1,400	1.33 (0.027)	0.068 (0.002)	19.6
1,900	1.52 (0.008)	0.008 (0.010)	18.6

Free-floating MULTITRAP design particle traps²⁶ were deployed approximately 100 km off the western coast of Mexico (17°8' N, 109°0' W) from 27 October to 17 November 1981. The light emission (integrated over 1 min) from a known volume of sediment-trap material, was recorded using an SAI model 2000 ATP photometer set at maximum sensitivity. Mean values ($n=3$) scaled to total trap volume are reported (± 1 s.d.). The phototube detector was calibrated using a standard light source (Optronic Laboratories, Inc. lamp 245 C) and a US National Bureau of Standards traceable photodiode (United Detector Technology silicon photodiode, PIN 10DSB). The spectrum of light from the lamp was regulated by a Melles Griot wide-band filter (03F1B003) with peak transmission at 440 nm and full-width half maximum of 85 nm. The specific bioluminescence measured from a luminescent bacterial culture, *Photobacterium leiognathi* strain 721 (ref. 27), at maximum light intensity ($A_{660}=0.54$) was 2.22×10^3 photons per cm^2 per s per pg ATP. Total sediment-trap ATP biomass was measured according to the methods of Karl and Knauer²⁸.

net plankton samples, whereas sediment-trap particles and faecal pellets characteristically showed a continuous and steady light output. The former response has been reported for certain species of dinoflagellates, copepods, euphausiids and planktonic larvae. The duration of these individual flash events is generally of the order of tens (or hundreds) of milliseconds¹⁸⁻²⁰. On the other hand, light emission from luminescent bacteria is continuous, even in single cells, and exhibits neither flashes nor oscillations²¹. Our observations suggest that enteric marine luminous bacteria are responsible for the light emission from faecal pellets, and possibly from materials collected in the sediment traps also. Recent data from a subsequent Vertex field experiment, near the same site, confirm this hypothesis. Luminescent bacteria have been isolated from sediment-trap materials and source-term faecal pellets and the *in situ* light emission has been shown experimentally to be of bacterial origin (C.C.A. and D.M.K., in preparation).

The bioluminescence of particulate matter collected in sediment traps was greatest at 30 m, diminished rapidly to less than one-third of this value by 120 m, and gradually decreased to a constant level with depth (Table 1). Interestingly, when light emission per unit biomass is examined (that is, 'biomass-specific' luminescence), the depth profile exhibits a distinct increase below 800 m (Table 1); this may have been a direct effect of the endogenous control of *in vivo* bioluminescence resulting from specific changes in environmental conditions such as oxygen or nutrient concentrations^{22,23}. The increase may also have been caused by an overall shift in the species composition favouring the succession of luminous bacteria at depth. A more likely explanation, however, is a direct relationship to the production and downward vertical flux of particulate matter. The observed increase in biomass-specific luminescence below 800 m correlates with the zone of enhanced biological activity and new particle production found just beneath the O_2 minimum (0.1–0.25 ml O_2 per litre). At 800 m, microbial rate processes associated with sediment trap-collected particles were maximal (D.M.K., unpublished) and within the water column, there was a substantial increase in the standing stock of nauplii and in total plankton dry weight (G. Knauer and M. Tuel, personal communication). Furthermore, stimulated *in situ* bioluminescence measured in the water column at the same station also showed increasing (but sporadic) activity below 700 m (ref. 24). These supplementary observations suggest that freshly produced pellets, at 800 m, may account for the greater particle bioluminescence at 900 m and below. It is conceivable that *in situ* measurements of bioluminescence, especially those which are associated with sinking particles, may be used for detecting the input of fresh organic detritus at intermediate oceanic depths.

The fact that copepod faecal pellets, as well as other particulate materials, are luminescent suggests an important relationship between feeding and light in the trophic structure of the mesopelagic realm. It also provides field support for the hypothesis that the functional importance of luminescence in marine bacteria^{22,25} is related to the dispersion and propagation of bacterial species. If faecal pellet microbial populations are generally inactive once the pellets are voided into the water, as suggested by this work, then bacterial decomposition would be insufficient for remineralization to occur during descent of the pellets. Slow decomposition combined with rapid settling rates would result in the loss of large quantities of carbon and energy-rich particles to the benthos. Direct feeding on these sinking bioluminescent particles by organisms from higher trophic levels may ensure against such loss and facilitate the propagation of bacterial species.

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Positional determination of supernumerary blast cell death in the leech embryo

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Segmented animals are divided into a longitudinal array of developmentally homologous subunits known as metameres. The embryonic origin of the segmental body plan has been studied in a variety of organisms, with particular emphasis on the mechanisms underlying the delineation of the individual metameres^{1–3} and their secondary diversification⁴. I have examined the embryonic events which determine the total number of segments in the glossiphoniid leech *Helobdella triserialis*. The germinal band of the leech consists of chains of segmental founder cells, called blast cells, and is normally reduced to 32 segments by the degeneration of supernumerary blast cells located at its caudal end^{5,6}. By using a novel technique for selective cell ablation, the segmental register of the four ectodermal cell lines can be altered so that lineally identifiable blast cells take part in the formation of ectopic segments. I show here that the survival or death of ectodermal blast cells is determined by position independent of their cell lineage identities, implying that the final number of segments is imposed on the ectoderm by interactions with the other embryonic tissues.

The egg of the glossiphoniid leech develops directly into the adult form through a stereotyped sequence of cell divisions^{7,8}, with the metamer portion of the ectoderm and mesoderm deriving from five bilateral pairs of blastomeres known as the M, N, O, P and Q teloblasts (Fig. 1). Each teloblast undergoes several dozen asymmetric divisions to generate a germinal bandlet—a chain of much smaller primary blast cells which will be designated here by the lower case letter corresponding to their teloblast of origin (Fig. 2a). On either side of the embryo the five bandlets come together in parallel to form a unified germinal band, with the firstborn blast cells lying at the future rostral end. By injecting the various teloblasts with vital cell lineage tracers and following the fate of the labelled clones^{9,10}, it has been shown that each teloblast gives rise to a specific, hemilateral set of segmentally iterated tissues in the mature leech^{8,11}. The present study focuses on the ectodermal bandlets o and p in which a single primary blast cell gives rise to one segmental complement of the tissues descended from its teloblast (D. A. Weisblat, in preparation).

The leech's segmental body plan can be envisioned as a two-dimensional array of blast cells in which one dimension—teloblast of origin—is related to the cellular composition of the descendent clone, while the second dimension—birth rank within the bandlet—dictates the rostrocaudal position, that is, the segmental fate of the clone. This strict correlation between a cell's genealogy and its fate suggests that individual blast cells follow distinct developmental pathways as a direct result of their cell lineage identities. The developmental importance of the teloblast of origin has been established by studies in which particular teloblasts were ablated by injecting them with toxic enzymes^{12,13}, although an instance of positional determination

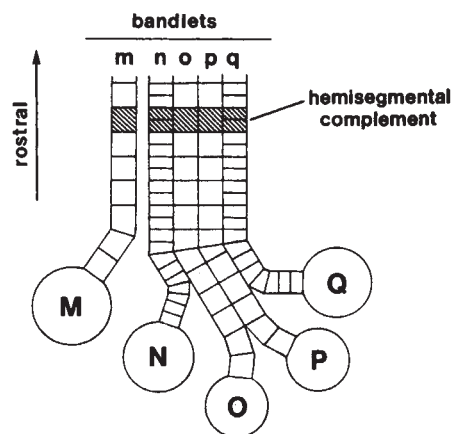


Fig. 1 Schematic diagram of germinal band formation in the leech embryo, taken from D. A. Weisblat (in preparation). The segmental tissues consist of five cell lines descended from the M, N, O, P and Q teloblasts. Each teloblast generates a bandlet of much smaller primary blast cells, and the five bandlets come together in parallel to form a unified germinal band. For the purposes of this illustration the mesodermal m bandlet has been displaced from its normal location beneath the four ectodermal bandlets. Each hemisegmental complement of the mature body wall and nervous system is founded by a homologous ensemble of seven primary blast cells.

has also been found¹⁴. To discover whether birth rank is responsible for segment-specific differences in blast cell development, I devised an experimental procedure involving the selective ablation of small groups of blast cells which cause cells in the remainder of the bandlet to take part in the formation of segments for which they are not normally destined.

Being too small for the reliable injection of toxic agents, blast cells were ablated by means of a photosensitizing lineage tracer inherited from their parental teloblast. This tracer, FDX, consists of fluorescein isothiocyanate covalently linked to a 10,000-molecular weight dextran carrier molecule which prevents passage across gap junctions. FDX is thus confined to the descendants of the injected teloblast and, in normal lighting conditions, serves as a vital cell lineage tracer¹⁰. However, FDX-labelled cells are dramatically sensitized to radiant energy within the fluorescein absorption spectrum¹⁵, and can be selectively ablated under an epifluorescence microscope using a $\times 40$ water immersion objective and a 50 W mercury arc with narrow band exciter filter (peak transmittance 485 nm). Labelled cells were safely viewed while orienting the embryos by shielding the light source to 1% maximal intensity. The iris was then closed so as to illuminate a circular spot of 15 μ m diameter, encompassing two primary blast cells, and the beam raised to maximal intensity for 20–60 s. This exposure bleaches a substantial fraction of the FDX fluorescence (Fig. 2b), and causes rapid degeneration of the labelled cells. The residual, unbleached tracer dissipates within 2–10 h after illumination (Fig. 2c), suggesting that the dying cells have lost their membrane integrity. In contrast, blast cells labelled with tracers which do not absorb at this wavelength can be illuminated for 300 s and still give rise to their normal descendent clones.

Photoablation of a patch of contiguous blast cells sunders the labelled bandlet into rostral and caudal fragments. If an o or p bandlet is lesioned just past the point where it merges into the germinal band, subsequent observations with low levels of illumination reveal that the caudal or trailing fragment is temporarily retarded from entering the band with a consequent widening of the gap produced by the lesion (Fig. 3a). The retarded bandlet slips out of its normal segmental register as the neighbouring bandlets slide past, and the constituent blast cells come to occupy segments for which they are not normally destined.

To demonstrate that blast cells are slipping into ectopic segments, the caudal fragment of the lesioned bandlet was com-

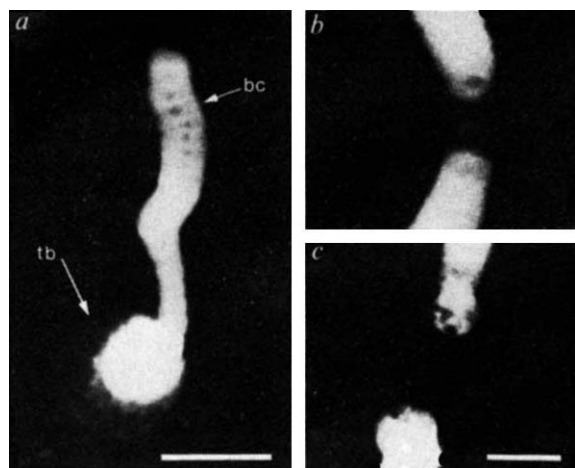


Fig. 2 Fluorescence micrographs of FDX-labelled germinal bandlets. *a*, The P teloblast (tb) has produced 20 labelled blast cell progeny (bc) 25 h after the injection of FDX. This tracer is excluded from the outer, yolky region of the teloblast cytoplasm, and from the blast cell nuclei. Scale bar, 50 μ m. *b*, Localized photobleaching of an FDX-labelled bandlet after 60 s exposure of two primary blast cells to intense 485-nm illumination. The target cells can be recognized by the pronounced photobleaching of their FDX fluorescence, and have already begun to display an abnormal, rounded morphology within minutes of illumination. *c*, The dying blast cells, seen here in a different embryo 5 h after illumination, lose their residual, unbleached tracer, suggestive of a breakdown in membrane integrity. Scale bar, 10 μ m.

pared with its unlesioned contralateral homologue by means of secondary tracer injections. In a normal embryo the simultaneous injection of bilaterally homologous teloblasts with the lineage tracer horseradish peroxidase (HRP) gives rise to an equal number of labelled segments on the two sides of the mature leech (Fig. 3*b*). If the right and left O or P teloblasts are injected with HRP after having produced 10–25 primary blast cells, the anterior boundaries of the right and left labelled regions fall within the same segment in the majority of cases (82%, 28/34) and never lie more than one segment apart. In a series of 24 experimental embryos the O or P teloblasts were initially injected with FDX, then simultaneously reinjected with HRP after the formation of a dozen or so singly labelled blast cells. On the right side of the embryo the first 2–3 FDX-labelled blast cells were ablated as they entered the germinal band. When these embryos were examined later in development, it was found that there was a sequence of several midbody segments containing both FDX and HRP on the control side, but only FDX on the lesioned side. On the average the anterior boundary of the HRP-labelled region was located six segments (range 0–12) more posteriorly on the lesioned side (Fig. 3*c*), demonstrating that blast cells in the caudal fragment of the lesioned bandlet had failed to reach their normal segmental destinations.

If segmental differences arise from autonomous properties of the individual blast cells, then slipped blast cells should develop in accordance with their birth rank despite their ectopic location. For the present study attention was focused on the process of supernumerary blast cell death. Each teloblast gives rise to 5–15 more blast cells than are necessary to populate the 32 body segments^{5,6}, and the excess blast cells degenerate—as judged by the dissipation of intracellular lineage tracers—during the construction of the germinal band. This pattern of development could be determined solely on the basis of cell lineage if each teloblast first generates the appropriate number of viable blast cells, and thereafter only produces supernumerary blast cells which differ in a way that brings about their premature destruction. This hypothesis was tested by examining blast cell death at the caudal end of slipped o or p bandlets (Fig. 4*a*). In 17 cases it was found that a bandlet which had slipped 3–10 seg-

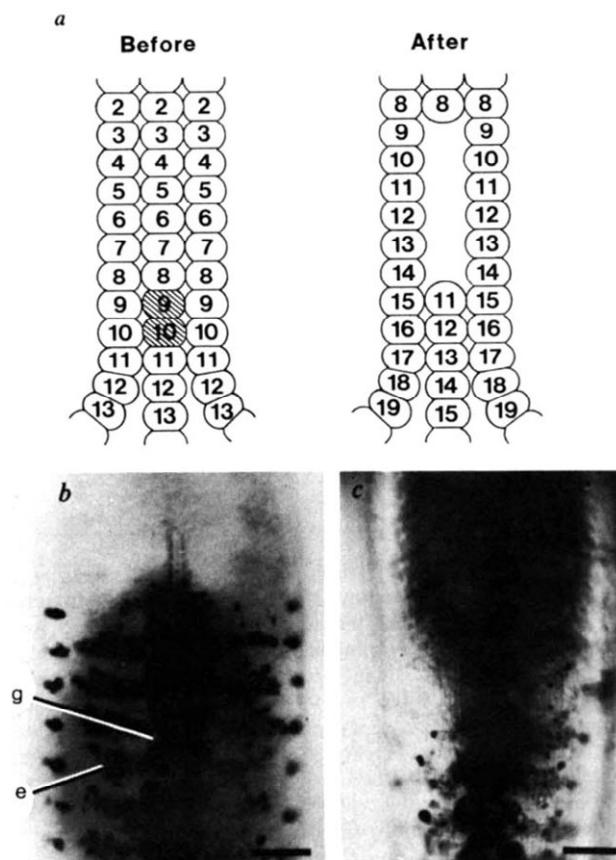


Fig. 3 Photoslesioned ectodermal bandlets slip out of their normal segmental register. *a*, Schematic diagram showing the point where the germinal bandlets merge into the germinal band. Numbers represent blast cell birth ranks and thus correspond to their segments of normal destiny. If one of the bandlets is lesioned (cross-hatching) near its point of entry, the trailing portion of the bandlet is temporarily retarded and thereby slips posteriorly with respect to the other, unlesioned bandlets. *b*, Stage 10 embryo in which the right and left O teloblasts were simultaneously injected with HRP during stage 7, midway through blast cell production. Anterior is at the top. Because homologous teloblasts produce their blast cell progeny in virtual synchrony, there are an equal number of labelled segments on the two sides. Note the segmentally iterated pattern of labelled tissues, including patches of body wall epithelium (*e*) and clusters of neurones within the segmental ganglia (*g*). *c*, In this embryo the right and left O teloblasts were simultaneously injected with HRP after the o bandlet on the left had been photolesioned near its rostral end, that is, well in advance of the first HRP-labelled cells. The disparity in the anterior boundary of the HRP-labelled region on the two sides demonstrates that the lesioned bandlet has slipped four segments posteriorly. Despite the change in longitudinal register the slipped blast cells have given rise to the normal pattern of descendant tissues within their ectopic segments. Scale bar, 50 μ m.

ments posteriorly began to degenerate at the same point (± 1 primary blast cell clone) as the other unlesioned bandlets (Fig. 4*b*). Similar results were obtained following slippage of lesioned n bandlets, and the combined slippage of ipsilateral o and p bandlets. Assuming that the lesioned bandlet(s) slips uniformly—as suggested by the segmental disposition of its descendant tissues (Fig. 3*c*)—then those blast cells which would normally have inhabited the tail segments have degenerated like supernumeraries on slipping past the posterior end of the germinal band.

These results suggest that ectodermal blast cells will degenerate, regardless of their birth rank, if they fail to become incorporated into the first 32 segments. Due to the unidirectionality of slippage it is unknown whether blast cells whose birth rank

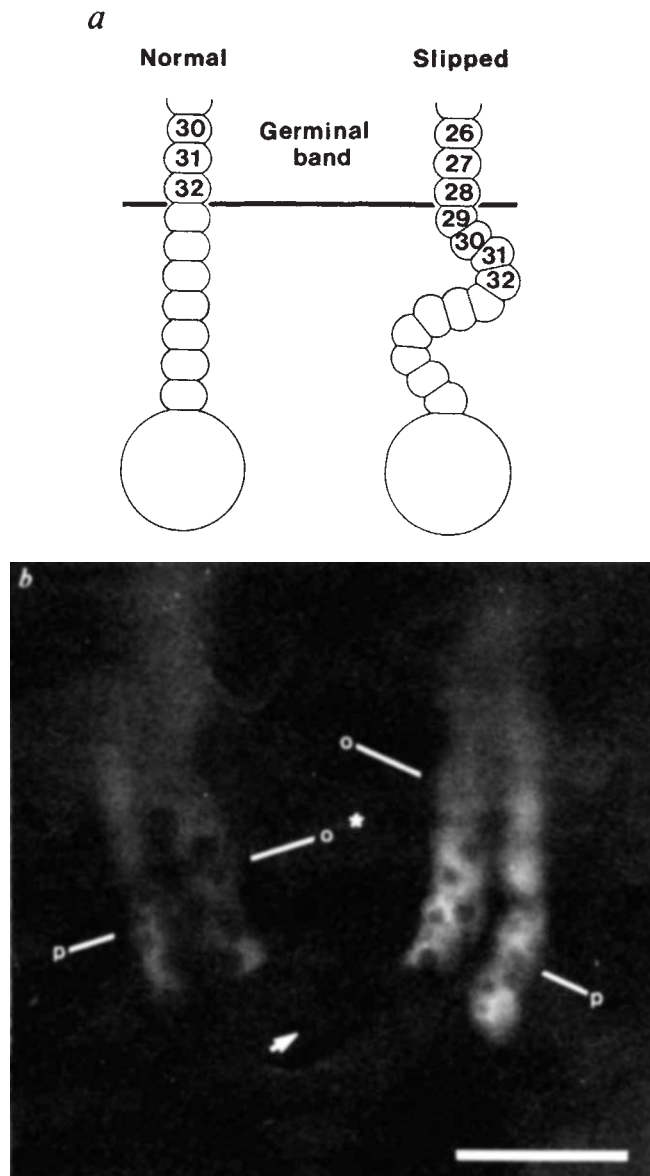


Fig. 4 Each teloblast produces several supernumerary blast cells which do not enter the germinal band and degenerate without giving rise to segmental tissue. *a*, If a lesioned bandlet slips posteriorly, those blast cells which would, on the basis of birth rank, normally inhabit the last of the 32 body segments will fail to enter the germinal band. *b*, Fluorescence micrograph of FDX-labelled in this stage 8 embryo the supernumerary blast cells have degenerated, leaving bits of fluorescent debris (arrow), and the incorporated blast cells have undergone secondary divisions to produce protosegmental clusters of daughter cells. The o bandlet on the left (labelled with an asterisk) was photolesioned near its rostral end and has slipped five segments posteriorly as measured by secondary tracer injection (not shown). This slipped bandlet terminates in register with the other, unlesioned bandlets, implying that position, not blast cell birth ranks, marks the point of transition between the surviving and dying blast cells. Scale bar, 50 μ m.

normally renders them supernumerary would survive and proliferate if they entered the germinal band. Still it would seem that blast cell death can be determined solely by the cell's rostro-caudal position with respect to the germinal band as a whole. Thus, supernumerary blast cell death is probably brought about by interactions with the cellular environment, as has previously been shown for the 'programmed death' of certain lineally identified cells in the nematode^{16,17}. Preliminary observations suggest that slipped ectodermal blast cells also exhibit host segment specificity in other aspects of their differentiation, for

example the fusion of the seven most caudal neuromeres to form a compound tail ganglion.

It is not yet known how the supernumerary blast cells detect their position relative to the end of the germinal band. For the hirudinid leech embryo it has been proposed that the point of ectodermal bandlet termination is determined by the underlying mesoderm⁶. However, ectodermal blast cells can survive past the stage of supernumerary blast cell death in mesoderm-deficient germinal bands¹². The role of the mesoderm was not examined in the present study because there was no apparent slippage following lesions of the m bandlet. Nonetheless, the present findings are consistent with the idea that the final length of the germinal band is fixed by some embryonic tissue or landmark other than the ectoderm, then subsequently imposed on the ectoderm by positionally determined death of the supernumerary blast cells.

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Inverse relationship between neurotensin receptors and neurotensin-like immunoreactivity in cat striatum

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The adult corpus striatum in mammals is divided into distinct histochemical compartments¹. If the cat caudate nucleus is stained for acetylcholinesterase a number of macroscopically visible zones appear that have lower acetylcholinesterase activity than the surrounding tissue². These patches, called 'striosomes'¹, correspond to regions of high [Met]-enkephalin-like immunoreactivity³ and dense opiate receptor binding⁴ and are related to the uneven distribution of striatal efferent neurones and cortical afferent terminations^{5,6}. One of the highest concentrations of neurotensin-like immunoreactivity is in the striatum and the immunoreactive material co-elutes with synthetic neurotensin on gel chromatography⁷. Recently, we have found that neurotensin-like immunoreactivity in the cat caudate nucleus coincides with the striosomes⁸. We have now localized neurotensin receptors in the cat caudate nucleus by autoradiography⁹ and found low density in the neurotensin-rich striosomes and a high density in the neurotensin-poor surrounding tissue.

Adult male cats received a lethal injection of pentobarbitone; their brains were removed and immersed in ice-cold saline for 10 min. Blocks containing the corpus striatum were frozen and sectioned in the transverse plane at 20 μ m using a cryostat. Adjacent sections were incubated with either 2 nM ³H-

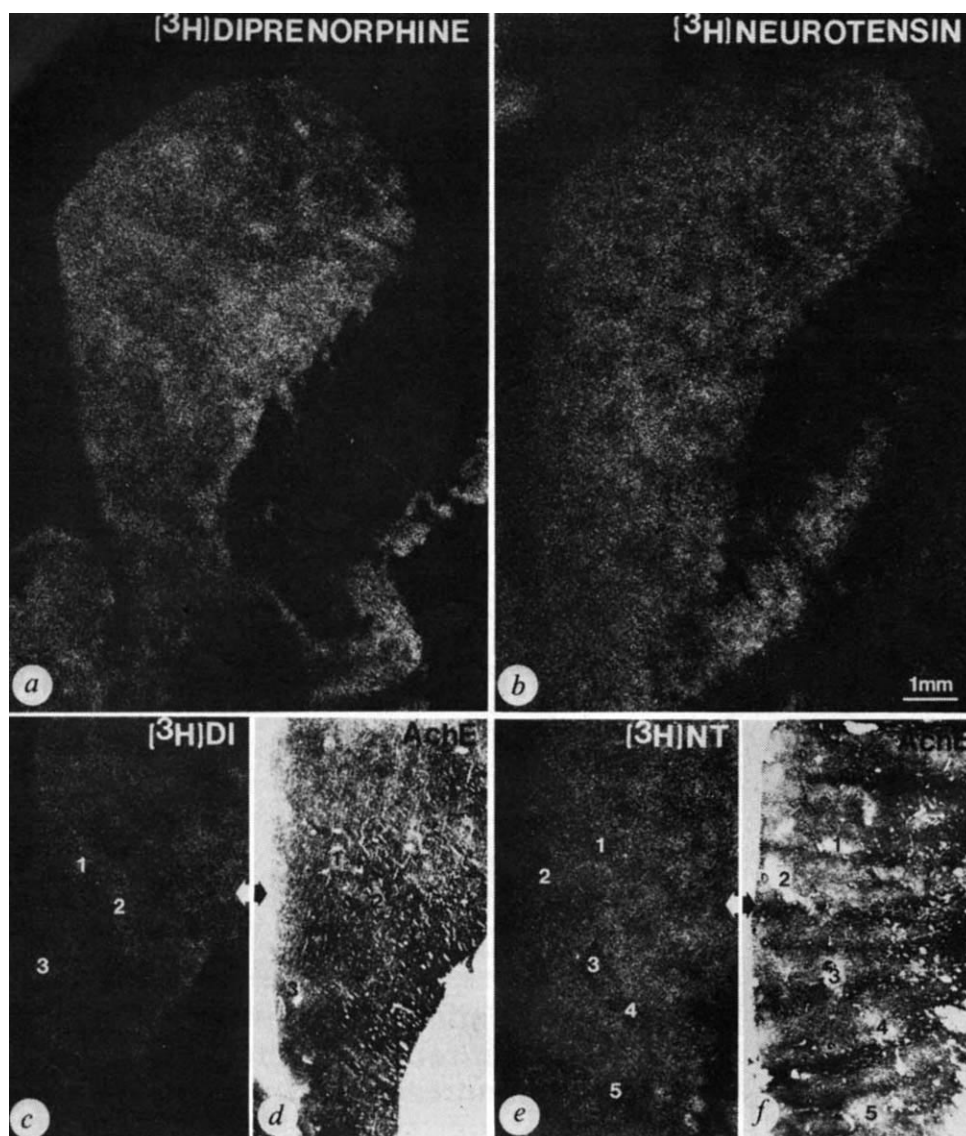


Fig. 1 Autoradiography of ^3H -neurotensin and ^3H -diprenorphine in the cat caudate nucleus. *a, b*, The caudate nucleus labelled with ^3H -diprenorphine and ^3H -neurotensin respectively; *c, d*, section labelled with ^3H -diprenorphine and stained for acetylcholinesterase; *e, f*, section labelled with ^3H -neurotensin and stained for acetylcholinesterase. The numbers in *c-f* refer to the acetylcholinesterase-poor striosomes. Note the correspondence between striosomes and dense ^3H -diprenorphine labelling and the inverse relationship between striosomes and ^3H -neurotensin binding sites.

Methods: Sections (20 μm) containing the cat striatum were pressed onto gelatin-coated slides, melted in place, dried overnight at 4 $^{\circ}\text{C}$ and stored at -20°C for 24 h. Control experiments had established that there was no detectable neurotensin- or [Met]enkephalin-like sections. The thawed sections were incubated for 10 min at 25 $^{\circ}\text{C}$ in 2 nM ^3H -neurotensin (New England Nuclear) in 50 mM Tris-HCl, pH 7.40, containing 0.1% bovine serum albumin, 40 mg l^{-1} bacitracin and 1 mM EDTA. Adjacent sections were incubated for 40 min at 25 $^{\circ}\text{C}$ in 4 nM ^3H -diprenorphine (Amersham), as described previously¹². Non-specific binding was estimated in parallel incubations in the presence of 1 μM neurotensin (Cambridge Research Biochemicals) or 1 μM naloxone (Endo). Following the incubation the sections were washed twice in ice-cold buffer (2 min each time), dipped into ice-cold water and dried under a stream of cold air. They were then arranged in X-ray cassettes, covered in the dark-room with a tritium-sensitive film (^3H -Ultrathin, LKB) and kept at -20°C for 6 weeks. Film was developed under safe-light conditions using Kodak D-19 developer. Tissue sections were stained for acetylcholinesterase¹⁰.

neurotensin or 4 nM ^3H -diprenorphine. Nonspecific binding was defined as binding in the presence of 1 μM neurotensin or 1 μM naloxone. The sections were apposed to a tritium-sensitive film and kept at -20°C for 6 weeks. The film was then developed and the tissue sections stained for acetylcholinesterase¹⁰. Test-tube binding of ^3H -neurotensin was performed using washed membranes from the cat caudate nucleus.

When sections of the striatum were incubated with ^3H -neurotensin an irregular distribution of silver grains was noted: whereas most of the caudate nucleus was labelled, local patches of sparse labelling occurred (Fig. 1*b*). No labelling was observed in the presence of 1 μM neurotensin. When the same sections were stained for acetylcholinesterase the regions rich in neurotensin binding sites (Fig. 1*e*) corresponded to acetylcholinesterase-rich regions (Fig. 1*f*). The neurotensin binding sites were further characterized using a test-tube binding assay for ^3H -neurotensin performed on washed membranes of the cat caudate nucleus. The binding was saturable (Fig. 2*a*) and Scatchard analysis indicated a single population of binding sites with a dissociation constant (K_D) of 1.54 nM and a maximum number of binding sites (B_{max}) of 68 fmol per mg protein (Fig. 2*b*). The competitive inhibition of ^3H -neurotensin binding by neurotensin appeared to be monophasic with a Hill coefficient not significantly different from 1 (Fig. 2*c*). The relative potencies of various neurotensin fragments in displacing

^3H -neurotensin were consistent with the potency values obtained for these fragments in biological assays¹¹ (Table 1), indicating that the binding properties of ^3H -neurotensin were consistent with the binding to a physiological neurotensin receptor. When sections of the cat striatum were incubated with ^3H -diprenorphine which labels all opiate receptor subtypes at the concentration used¹², local patches of dense labelling embedded in a less dense background were observed (Fig. 1*a*). This labelling pattern was completely abolished in the presence of 1 μM naloxone. When stained for acetylcholinesterase the regions rich in ^3H -diprenorphine receptors (Fig. 1*c*) coincided with the acetylcholinesterase-poor striosomes (Fig. 1*d*). The results obtained by histochemistry and receptor autoradiography were further evaluated using microdensitometry (Table 2). The neurotensin- and [Met]-enkephalin-like immuno-reactivities were six times more concentrated in the striosomes than in the surrounding tissue, whereas the neurotensin receptors were higher by 25% in the tissue around the striosomes and the opiate receptors 20% more numerous within the striosomes. The acetylcholinesterase staining was 30% higher in the non-striosomal tissue.

The present findings indicate that neurotensin receptors are unevenly distributed throughout the caudate nucleus of the adult cat with areas of dense labelling corresponding to acetylcholinesterase-rich regions. This contrasts with

Table 1 Inhibition of specific ^3H -neurotensin binding to cat caudate nucleus membranes by neurotensin fragments

Peptide	IC ₅₀ (nM)	Relative potency %
NT ₁₋₁₃	2.80	100
NT ₈₋₁₃	2.60	107
NT ₉₋₁₃	265	1.1
NT ₁₀₋₁₃	>10,000	<0.04
NT ₁₋₆	>10,000	<0.04
NT ₁₋₈	>10,000	<0.04
NT ₁₋₁₁	>10,000	<0.04

The binding of tritiated neurotensin (NT) was assayed at a concentration of 2 nM. IC₅₀ was defined as the concentration of unlabelled peptide required to inhibit the specific binding of ^3H -neurotensin by 50% and it was derived from the Hill transformation of the displacement curves. Similar IC₅₀ values were obtained in three separate experiments. The relative potencies were calculated by comparing the IC₅₀ values with that of NT₁₋₁₃. NT₉₋₁₃ and NT₁₀₋₁₃ were synthesized by Dr B. Williams (MRC Neurochemical Pharmacology Unit), NT₈₋₁₃ was purchased from Peninsula Laboratories and all other peptides were obtained from Cambridge Research Biochemicals.

Table 2 Relative distribution of acetylcholinesterase activity, [Met]-enkephalin-like immunoreactivity, neurotensin-like immunoreactivity, opiate receptors and neurotensin receptors in striosomes and background matrix of the cat caudate nucleus

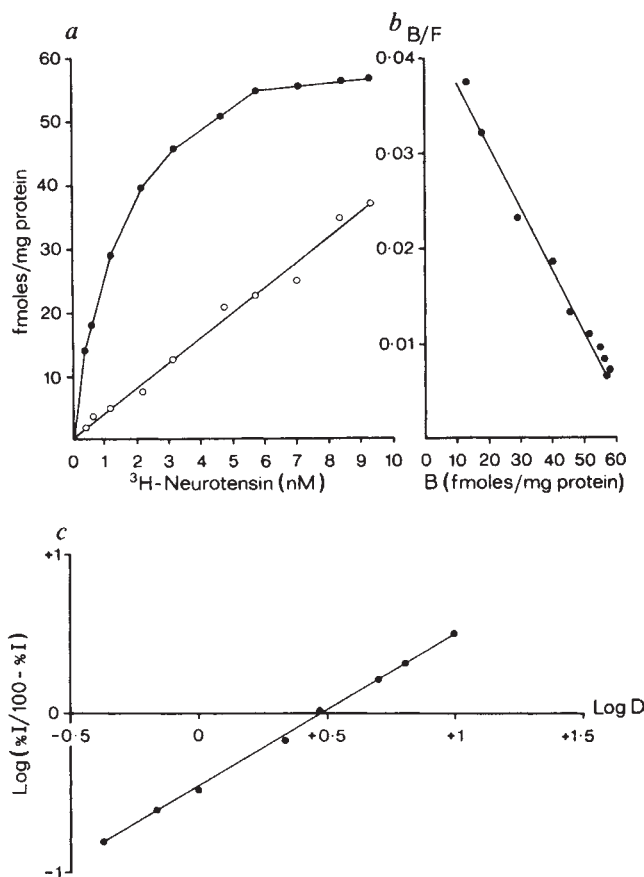
	Striosomes	Background matrix
Acetylcholinesterase	100 ± 3.10%	129.55 ± 4.38%*
[Met]-enkephalin-like immunoreactivity	100 ± 6.18%	14.00 ± 3.77%*
^3H -diprenorphine binding	100 ± 4.77%	81.41 ± 5.01%†
Neurotensin-like immunoreactivity	100 ± 11.52%	16.12 ± 7.05%*
^3H -neurotensin binding	100 ± 0.85%	125.75 ± 1.81%*

Striosomes and background matrix of the dorsal anterior part of the cat caudate nucleus (where the striosomal organization is the most marked) stained for acetylcholinesterase, [Met]-enkephalin- or neurotensin-like immunoreactivity⁸ and incubated with ^3H -diprenorphine or ^3H -neurotensin were quantitatively assessed by using microdensitometry, as described previously²¹. The intensity in staining in the striosomal region was arbitrarily defined as 100% and the staining in the matrix expressed relative to it. Background staining for the immunohistochemistry and receptor binding was obtained from adjacent absorption control sections and subtracted for both striosomes and background matrix. For acetylcholinesterase background was taken to be the staining present in the internal capsule. The results represent the mean ± s.e.m. ($n = 5$).

* $P < 0.001$; † $P < 0.05$.

neurotensin-like immunoreactivity which is distributed like the previously described striosomes⁸ defined by a low acetylcholinesterase activity and a high content of enkephalin-like immunoreactivity¹. In the rat⁴, opiate receptors coincide with [Met]-enkephalin-like immunoreactivity in the caudate nucleus. Lesion studies have indicated that rat opiate receptors are located on both intrinsic striatal neurones and dopamine-containing terminals, the receptor patches being mainly located in striatal neurones^{13,14}. Similarly, we have found that 60% of rat striatal neurotensin receptors are localized on neurones intrinsic to the striatum¹⁵.

The reason for the inverse relationship between neurotensin receptors and neurotensin-like immunoreactivity is unknown at present. Possibly, it could arise from different rates of turnover of neurotensin-like immunoreactivity in striosomes as compared to acetylcholinesterase-rich regions. Such regional differences have been observed for the turnover of dopamine in the rat striatum¹⁶. Discrepancies between the localization of either classical neurotransmitters or neuropeptides and their respective

**Fig. 2** ^3H -neurotensin binding to a membrane preparation of the cat caudate nucleus. *a*, ^3H -neurotensin binding as a function of the radiolabelled peptide concentration. Values were obtained in a single experiment. Similar results were obtained in four separate experiments. *b*, Scatchard plot of specific ^3H -neurotensin binding data. *c*, Hill transformation of ^3H -neurotensin binding by increasing concentrations of unlabelled neurotensin. A typical experiment is shown; similar results were obtained in four separate experiments.

Methods: The caudate nucleus from adult cats was homogenized in 10 volumes (w/v) of ice-cold 50 mM Tris-HCl, pH 7.40, with a Polytron at setting 7 for 15 s. After 20-min centrifugation at 50,000g the pellet was resuspended in 10 volumes of 50 mM Tris-HCl, incubated at 37 °C for 30 min and centrifuged again. The washed pellet was resuspended in 50 mM Tris-HCl, pH 7.40, containing 0.1% bovine serum albumin, 40 mg l⁻¹ bacitracin and 1 mM EDTA to yield a concentration of 10 mg tissue per ml buffer. One ml of homogenate was incubated in the presence of ^3H -neurotensin (New England Nuclear) at 25 °C for 10 min. Non-specific binding (○) was defined as binding in the presence of 1 μM neurotensin and specific binding (●) was obtained by subtracting the nonspecific from the total binding. At the end of the incubation period the tubes were transferred onto ice and filtered immediately under reduced pressure through GF/B glass fibre filters (Whatman) pretreated with 0.2% polyethyleneimine (Sigma) in water. Each of the filters was washed four times with 5 ml ice-cold buffer and radioactivity determined by liquid scintillation spectrometry. A detailed characterization of the binding assay will be published elsewhere²². Proteins were determined according to Lowry *et al.*²³ using bovine serum albumin as the standard.

receptors have been noticed before. For instance, there exists a mismatch between noradrenaline levels and beta-adrenergic receptor numbers in the rat striatum^{17,18} and between the content of substance P-like immunoreactivity and the number of substance P receptors in the rat substantia nigra^{19,20}. Our present finding that neurotensin-like immunoreactivity and neurotensin receptors have different topographical localizations within an anatomical structure characterized by distinct histochemical compartments may have general implications for the function of basal ganglia.

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Suppression of sprouting at the neuromuscular junction by immune sera

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Injury of afferent motor axons or pathological loss of motoneurons from the spinal cord causes the remaining axons within a muscle to sprout and to reinnervate the denervated muscle fibres. Sprouting occurs at two sites along intramuscular axons, at nodes of Ranvier (nodal sprouting) and at the neuromuscular junction (terminal sprouting)^{1,2}. Terminal sprouting is also produced by treatment with botulinum toxin and by other agents that render muscle inactive³⁻⁷. The muscle probably provides a signal for terminal sprouting as restoration of muscle activity by direct electrical stimulation prevents sprouting⁸⁻¹⁰. Such a signal might be a local change on the muscle fibre surface or a 'soluble' sprouting factor^{2,4}, although the failure to induce terminal sprouting in one muscle by denervating adjacent muscles argues against the latter hypothesis¹¹⁻¹³. I now report that rabbit antisera against a 56,000 (56K)-molecular weight protein secreted by denervated rat muscle suppress botulinum toxin-induced terminal sprouting in the mouse gluteus muscle. An immune response against this protein has also been detected in serum of patients with amyotrophic lateral sclerosis (ALS), a disease in which loss of motoneurons from the spinal cord is not accompanied by the degree of sprouting and reinnervation seen in other motoneuron diseases¹⁴.

In 1977, Lømo, Brookes and Raff produced a rabbit antiserum against dialysed medium conditioned by an organ-cultured rat hemidiaphragm that was freshly denervated by being placed in culture. An IgG fraction from the antiserum thus obtained was used to construct an immunoaffinity column over which was passed medium conditioned (DdiaCM) by a hemidiaphragm that

Table 1 Effects of antisera on botulinum toxin-induced terminal sprouting in the mouse gluteus

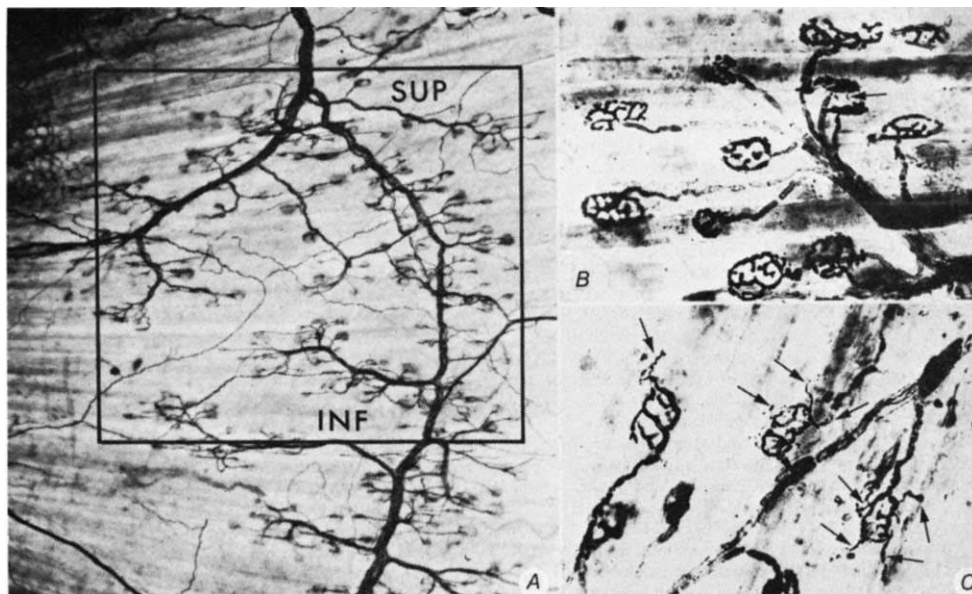
Serum	Toxin	SP%	NSP%	P (SP%)	P (NSP%)
D6pre	0712	55 ± 5	101 ± 9	NS	NS
D10pre	0911	42 ± 9	84 ± 18	NS	NS
D8	0712	43 ± 5	79 ± 9	NS	0.05
D5	0630	60 ± 6	105 ± 10	NS	NS
D6	0630	56 ± 7	98 ± 13	NS	NS
D7	0401	17 ± 3	36 ± 6	0.0001	0.0001
D9	0712	33 ± 4	61 ± 8	0.005	0.0005
D10	0910	32 ± 4	68 ± 8	0.005	0.005
D11	0823	28 ± 3	69 ± 7	0.0005	0.005

The data base consisted of the nine groups of eight mice each listed (D6pre and D10pre were pre-immune sera), six groups of mice that received sera from neurologically normal human subjects, six groups that received sera from patients with diabetic peripheral neuropathy and 19 groups that received sera from sporadic ALS patients. The data for the human sera tested are reported in detail elsewhere²⁰. One-way analysis of variance (Statistics Package for the Social Sciences²⁸) was used to establish that within each control treatment the distribution of SP% (% of axon terminals with sprouts) for the mice within individual groups did not differ (toxin alone, pre-immune rabbit, normal human, diabetic), and that the distribution of mean SP% for the groups within the different control treatments did not differ across the treatments (d.f. 3, 14; $F = 1.189$; $P = 0.35$). Thus, the controls were homogeneous and the data could be pooled. The distribution of SP% for the pooled data (138 muscles) was normal (χ^2 goodness of fit test) with mean 47.9%, σ 16.1%. To assess which sera significantly suppressed sprouting, the z-score for each experimental group was tested against the parameters of the normal distribution for SP% of the pooled controls. During the study, six dilutions of the 10 μ g ml⁻¹ toxin stock were used (listed by date in the second column). The SP% averaged among control groups that received each dilution did not differ significantly (F -test), but ranged from 41 to 57%. To remove this source of variation, the mean SP% for each group was normalized against the average SP% for the control groups receiving the same dilution of toxin (NSP%). The parameters of the distribution of NSP% for the pooled controls were mean 100%, σ 33%. The probabilities of the calculated z-scores for SP% and NSP% are given. Values shown are $\pm$ s.e. NS, not significant.

had been denervated 4 days *in vivo* before being placed in culture. The material that passed through the column was used to produce a second antiserum. In 1981, preliminary results obtained by Kemplay and Stolkin suggested that the second antiserum suppressed partial denervation-induced terminal sprouting in the rat sternocostalis muscle by about one-half (personal communication). I obtained a similar effect using the rat fourth deep lumbrical muscle (denervation protocol as in ref. 5). None of the first antiserum remained for testing.

To produce a second blocking antiserum, I fractionated the DdiaCM protein by gel filtration and took two size fractions for immunization (see Fig. 2 legend). Material eluting in the void volume of the column was used to immunize two rabbits (D5 and D6), and material eluting at ~60-40K was used to immunize a second pair (D7 and D9). These molecular weight ranges were chosen on the basis of preliminary immunoblot analysis of the antigens in the DdiaCM reacting with the blocking antiserum produced by Lømo, Brookes and Raff. A fifth rabbit (D8) was immunized with the unfractionated DdiaCM. The five antisera were tested for suppression of botulinum toxin-induced terminal sprouting in the mouse gluteus muscle (see Fig. 1 legend). Only antisera from D7 and D9 significantly suppressed sprouting (by 60% and 40%, respectively; see Table 1). D7 antiserum also suppressed terminal sprouting in C5-deficient, DBA/2NCo mice (Laboratory Supply Co.), but failed to suppress sprouting if delivered intraperitoneally (i.p.) rather than subcutaneously (s.c.) over the surface of the gluteus. Immunoblot analysis of the antigens in the DdiaCM reacting with the various antisera revealed that an antigen of molecular weight ~56K reacted with the D7 and D9 antisera, but not with any of the non-blocking antisera (Fig. 2). The antigen was a minor component of the DdiaCM which was not visualized in Coomassie blue-stained gels. To purify this antigen for immunization, 1 mg of

Fig. 1 A, Whole mount of the mouse gluteus muscle stained by a combined silver–cholinesterase technique²³ to indicate details of its innervation. The gluteus was chosen for the sprouting assay as both toxin and experimental antisera could be injected directly over the surface of the relatively thin sheet of muscle fibres. Also, the muscle could be viewed in whole mount, which left the terminals undisturbed by the dissection (magnification $\sim \times 35$). B, Zinc iodide–osmium tetroxide (Z10)-stained nerve terminals in a botulinum-treated muscle exposed to an antiserum (D7) that suppressed terminal sprouting. C, Same as B but exposed to an antiserum (D8) having no effect. Fine processes are seen sprouting from some of the axon terminals (arrows in B and C; magnification $\sim \times 190$). Except for the presence of fewer sprouting terminals in B than in C, no other histological differences were apparent in the Z10-stained material. The superior (SUP) and inferior (INF) gluteal nerves can be seen to meet across the midsection of the muscle in A. In each muscle examined, the SP% (see Table 1) was determined in the zone of innervation of the superior gluteal nerve within the region delimited by the box. An average of 100 terminals was scored for sprouting in each muscle.



Methods: To induce terminal sprouting, female ICR mice (Sprague–Dawley, 20–24 g) were injected s.c. over the surface of the right gluteus muscle with ~ 250 pg of botulinum toxin type A (Sigma) in $5 \mu\text{l}$ of $70 \text{ mM NaH}_2\text{PO}_4$, 0.2% gelatin buffer ($\text{pH } 6.5$). The toxin was diluted from a $10 \mu\text{g ml}^{-1}$ stock and titred by injecting five groups of eight mice each with 62.5, 125, 250, 500 and 800 pg of toxin, respectively. The index of terminal sprouting (SP%) increased linearly over the 62.5–250 pg doses (from $25 \pm 4\%$ to $49 \pm 4\%$). The 500 and 800 pg doses produced 37% and 75% lethality, respectively, while the 250 pg dose chosen for the study produced only 4% lethality overall (512 mice injected). Visible signs of paralysis were flaccidity of the ipsilateral abdominal wall and diminution of the toe spreading reflex in the injected leg. On each of the 6 days after toxin injection, groups of eight mice were injected s.c. over the gluteus with 0.1 ml of a heat-inactivated (60°C , 30 min) test serum. Groups of mice injected with toxin alone, or that received the series of injections with serum, were killed 7 days after toxin injection. The gluteus was excised and stained with Z10²⁴ for 3 h in the dark at 23°C . Muscles were cleared in xylene and whole-mounted in Permount. Before microscopic examination ($\times 250$ magnification with a Leitz Pl Apo 25/0.65 objective), slides were randomly coded in batches of 40–80 (from 5–10 experimental groups). Data for six muscles that stained too darkly and six that stained too lightly were eliminated from the study before decoding (out of 488 muscles examined); no other selection of the data was made.

protein from the 60–40K AcA-44 fraction was separated on a 7.5% SDS-polyacrylamide gel (see Fig. 2 legend). After brief staining with Coomassie blue to visualize the major proteins in the fraction, the region of ~ 60 – 54K was excised from the gel, homogenized in saline, then used to immunize another pair of rabbits (D10 and D11). Antigenic material was recovered from the gel as both rabbits developed antisera that suppressed terminal sprouting (Table 1). Both D10 and D11 antisera reacted with the 56K antigen on immunoblotting, although neither was monospecific (Fig. 2). D11 reacted with some poorly resolved material of 66K and 60K which also reacted with D9, but not with D7 or D10. D10 reacted with some material of $\sim 63\text{K}$ which also reacted with D7, but not with D9 or D11. Thus, the 56K antigen still seemed the most likely candidate for the antigen of interest. The other material, but not the 56K antigen, was also detected in immunoblots of rat serum reacted with D10 or D11.

Clinical studies have been unable to determine whether the decreased reinnervation seen in ALS is an active process of the disease, or simply due to motoneurone death outpacing the rate of reinnervation^{14–19}. Evidence that ALS sera suppressed terminal sprouting would favour the first hypothesis. To test this, serum samples were obtained from 19 sporadic ALS patients with both upper and lower motoneurone signs and tested for suppression of sprouting in the mouse gluteus assay. Sprouting was significantly suppressed by 8/19 ALS sera (NSP%, $P < 0.05$; see Table 1 legend), but not by six sera from healthy individuals²⁰. The three most effective sera (N.L., R.V. and E.Z.; NSP%: $9 \pm 2\%$ ($\pm \text{s.e.}$), $47 \pm 10\%$, $71 \pm 10\%$, respectively) were reacted with immunoblots of the rat DdiaCM. Each reacted with the rat 56K antigen and little else in the DdiaCM, while none of the six normal human sera reacted with the antigen

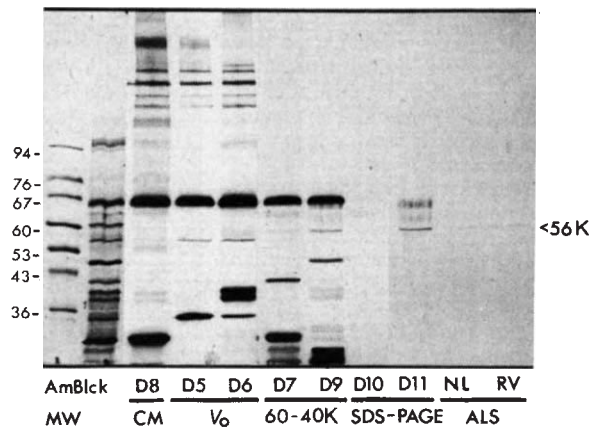
(data for N.L. and R.V. are shown in Fig. 2). These data provide evidence for active suppression of sprouting in some cases of ALS. The detection of antibody against the rat 56K antigen in several blocking ALS sera further implies that the disease may have an autoimmune component directed against a human equivalent of the rat antigen, and provides independently derived evidence corroborating the results obtained with the experimental antisera.

Due to the polyclonal nature of the sera used in the study, identification of the 56K antigen as the effective antigen for eliciting a blocking antiserum has not been unequivocally established—that will require the use of a monoclonal reagent, which is now being produced. Nor can the biological role of the 56K antigen be defined with the data in hand. However, the molecular weight of the antigen on gel filtration (60–40K) and on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is similar to that reported for activities in conditioned medium that stimulate neurite outgrowth from spinal cord explants²¹ and dissociated spinal cord neurones²². Preliminary experiments have shown that the 56K antigen co-purifies 100–200-fold with an activity(s) that promotes sprouting of neurites and enhances survival of dissociated spinal cord cells in culture, but elucidation of the antigen's biological activity awaits its final purification.

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Fig. 2 Immunoblot analysis of the sera described in the text. Molecular weight markers were loaded in the lane marked MW and the other lanes were loaded with 35 µg of protein obtained from medium conditioned by a denervated rat hemidiaphragm (DdiaCM). The first two lanes of the filter were stained for protein with amido-black, the rest were allowed to react individually with one of the sera described in the text. Bound γ -globulin was detected with 125 I-labelled *Staphylococcus* protein A and the autoradiogram of the filter is shown. The antisera produced in rabbits D8, D5 and D6 failed to suppress terminal sprouting, while those from D7, D9, D10 and D11 all exhibited suppression. Sera from two ALS patients (N.L. and R.V.) also suppressed terminal sprouting. All sera that suppressed terminal sprouting reacted with an antigen of ~56K (56.6 ± 0.5 K, $\pm$ s.d. for three determinations) which is believed to be the effective antigen in the immunizations.

Methods: The left hemidiaphragms of male Sprague-Dawley rats (150–200 g) were denervated by cutting the phrenic nerve under ether anaesthesia, and cultures were prepared as described by Merlie *et al.*²⁵, except that no serum was added to the medium. The medium consisted of Dulbecco's modified Eagle's medium (Flow Labs) supplemented with 33 µg ml⁻¹ bovine insulin (Sigma) and 35 µg ml⁻¹ *p*-aminobenzoic acid with buffering maintained by bicarbonate in an atmosphere of 5% CO₂ in O₂ at 37°C. The hemidiaphragms were pinned out on Sylgard (Dow Corning) slants poured in 100-mm Petri dishes and were rocked twice per min. After 2–4 h in culture, the medium was replaced, then collected after a further 24 h (DdiaCM). Protein content ranged from 1 to 2 mg ml⁻¹ (Bio-Rad protein assay; horse γ -globulin protein standard). The DdiaCM was stored frozen at -20°C for up to 6 months. Antisera were produced in female SPF New Zealand White rabbits (12–14 kg). D8 antiserum was raised against the dialysed (50 mM NaH₂PO₄, 1 mM Na₂EDTA, pH 7.5, 4°C), unfractionated DdiaCM. To fractionate the DdiaCM by gel filtration for immunization of D5, D6, D7 and D9, 100 ml of DdiaCM (45 mg protein) were dialysed against 0.1 M NaH₂PO₄, 0.2 M NaCl, 5 mM Na₂EDTA (pH 7.5) at 4°C, concentrated by ultrafiltration to 6 ml (Amicon stirred cell, YM-10 membrane), then applied to a 2.5 × 100 cm column packed with AcA-44 (LKB) and eluted in the same buffer at a flow rate of 17.5 ml hr⁻¹; 8-ml fractions were collected. The column was calibrated with thyroglobulin to indicate the void volume (V₀), bovine serum albumin (67K), ovalbumin (44K), and carbonic anhydrase (29K). Fractions containing material that eluted in the V₀ (15.6 mg protein) were pooled as were fractions eluting between 60 and 40K (6.3 mg protein). The two pooled fractions were dialysed against 20 mM ammonium bicarbonate, then lyophilized. Rabbits D5 and D6 were immunized with the V₀ AcA-44 fraction, while D7 and D9 received the 60–40K AcA-44 fraction. For immunization, 1 mg of protein (dry weight) was dissolved in phosphate-buffered saline (PBS; 5 mM NaH₂PO₄, 0.9% NaCl, pH 7.5) and emulsified in an equal volume of adjuvant. Freund's complete adjuvant was used for the first immunization (10–15 intradermal sites) and in a second immunization at 2 weeks. After 6 weeks, booster injections were given in incomplete adjuvant; blood was obtained two weeks after each boost. After collection, blood was clotted, the serum separated, heat-inactivated at 60°C for 30 min, then stored frozen at -20°C in 1-ml aliquots for use in the sprouting assay. To purify antigen by preparative SDS-PAGE for the D10 and D11 immunizations, 1 mg of the lyophilized 60–40K AcA-44 fraction was boiled for 2 min in 10% (w/v) glycerol, 0.08 M Tris-HCl pH 6.8, 2.0% (w/v) SDS, 5.0% (v/v) β -mercaptoethanol and electrophoresed through a 1.5 mm-thick, 7.5% SDS-polyacrylamide gel²⁶. The gel was briefly stained in Coomassie blue (0.125% in 10% acetic acid, 50% methanol) then destained in 10% acetic acid, 15% methanol. The 60–40K AcA-44 fraction contained the prominent doublet at ~60K seen in the DdiaCM stained with amido-black, as well as the next prominent band of ~54K. The region between these two markers was cut from the gel, briefly rinsed in several changes of PBS, minced, then Dounce-homogenized in 1–2 ml of PBS. The gel slurry was emulsified in Freund's complete adjuvant for the first immunization, then in incomplete adjuvant for immunizations at 10 and 13 weeks. D10 was exsanguinated at 17 weeks. D11 was bled at 15 weeks, at which time it exhibited muscle wasting and had lost about one-third of its body weight; this rabbit died 1 week later and the carcass was unintentionally destroyed. All of the other rabbits remained healthy and a muscle biopsy of D7 at 10 weeks showed no signs of denervation (K. Stefansson, data not shown). For immunoblotting, 35 µg of DdiaCM protein in culture medium was boiled in the above SDS sample buffer and electrophoresed through a 1.5 mm-thick, 7.5% polyacrylamide (bis, 2.5% w/w of total monomer) slab gel²⁶ with cooling. The protein was electrophoretically transferred²⁷ from the gel to a nitrocellulose filter (BA85; Schleicher and Schuell) at 3.5 V cm⁻¹, 0.2 A for 1 h. After transfer, staining of the trichloroacetic acid-fixed gel with Coomassie blue demonstrated complete transfer of all protein having a molecular weight <94K. Individual lanes were cut from the filter and stained for protein with amido-black (0.1% in 10% acetic acid, 50% methanol) or soaked in 5% haemoglobin in PBS-Na₃ for 1 h to block reactive sites. The 'blocked' strips were reacted overnight at 23°C with sera diluted 1:50 in 1% haemoglobin. IgG bound to the filter was detected by incubation for 3 h with *Staphylococcus* protein A (Sigma) iodinated by the chloramine-T method to 5–10 µCi per µg protein, followed by exposure to Kodak XRP-5 film overnight at 4°C. Molecular weight markers (Sigma) were: phosphorylase B (94K), human transferrin (76K), bovine serum albumin (67K), catalase (60K), glutamic dehydrogenase (53K), ovalbumin (43K) and lactate dehydrogenase (36K).



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Myelin-associated glycoprotein in human retina

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The human retina is unmyelinated, but structural similarities have been noted between Müller cells, the main glial cell type of retina, and oligodendrocytes, the myelin-forming cells of the central nervous system¹. We now show that antibodies against myelin-associated glycoprotein, a minor component of central and peripheral myelin so far found only in myelin and myelin-forming cells^{2–4}, also stain Müller cells. Immunoblot analysis of retinal proteins indicates that the antigen detected is myelin associated glycoprotein. These results suggest a closer relationship between Müller cells and oligodendrocytes than previously suspected and raise questions about the functional role of myelin-associated glycoprotein.

Rat monoclonal antibodies against myelin-associated glycoprotein were obtained as a by-product of attempts to raise monoclonal antibodies specific for membrane-bound nicotinic acetylcholine receptor (AChR) from the electric organ of *Torpedo californica*. Rats were immunized with AChR from the electric organ, tissue known to be rich in myelinated axons. The preparation of acetylcholine receptor used for the immunization was later shown to be contaminated with myelin. The monoclonal antibodies were raised by fusing spleen cells from the immunized animals to the non-secreting mouse myeloma cell line SP2/O-Ag14 using the hybridoma technique of Köhler and Milstein⁵, slightly modified⁶. The hybridoma supernatants were screened against AChR-containing membrane preparations from the electric organ using an enzyme-linked immunoassay⁷. Two IgG monoclonal antibodies produced in this way (BRIC₃₇ and BRIC₃₈) that did not bind to the acetylcholine receptor reacted on a Western blot⁸ with isolated human MAG (given by Dr R. H. Quarles). When Western blots of homogenates of adult human spinal cord or human peripheral nerves were studied, the antibodies reacted only with bands having relative mobilities identical to that of MAG (Fig. 1). The monoclonal antibodies showed no reaction with Western blots of homogenates from human liver, kidney or striated muscle.

The immunohistochemical distribution of the antigen with which BRIC₃₇ and BRIC₃₈ react is identical to that described for MAG^{3,4}. The antigen is present in myelin sheaths of the central and peripheral nervous systems as well as in the cytoplasm of oligodendrocytes just prior to and during myelination. As controls for BRIC₃₇ and BRIC₃₈, both on the Western blots and in immunohistochemical work, we used several monoclonal antibodies against the nicotinic AChR⁶. We also used serum from a patient (M) with IgM monoclonal gammopathy and neuropathy whose monoclonal IgM is directed against human MAG⁹ (Fig. 1). As controls for this serum we used sera from several patients with IgM monoclonal gammopathy unaccompanied by neuropathy. It has been demonstrated by peptide mapping that BRIC₃₇ and BRIC₃₈ may react with the same epitope on MAG but one which differs from that which reacts with IgM from the patient's (M) serum (L.S.M. and K.S., in preparation).

Sections of formalin-fixed, paraffin-embedded human retinas obtained from autopsy and surgical material were stained immunohistochemically with BRIC₃₇ and BRIC₃₈ using the peroxidase-antiperoxidase (PAP) method of Sternberger¹⁰. They were also stained with the patient's serum described above, using the biotin-avidin peroxidase technique¹¹. There was intense staining of processes throughout the retina, reaching down to the inner limiting membrane and up to the outer limiting membrane (Fig. 2). At the outer limiting membrane there was staining of the fine processes of the Müller cells that reach between the inner segments of the photoreceptors. A few nuclei in the inner nuclear layer were outlined by a darkly staining rim (Fig. 3). These nuclei were located where nuclei of Müller cells are known to be present. This staining pattern is similar to that described for monoclonal antibodies raised against Müller cells¹². The controls gave no staining (Fig. 2).

Fresh normal human retinas were homogenized, solubilized, electrophoresed on a polyacrylamide gel, and blotted onto nitrocellulose paper as described previously⁹. The nitrocellulose paper was subsequently exposed to BRIC₃₇, BRIC₃₈ or the patient's (M) serum. The anti-MAG antibodies identified a band having a relative mobility identical to that of MAG (molecular weight (M_r) ~100,000), another band of ~130,000 M_r , a third at ~200,000 M_r and a few minor bands of larger molecular weights (Fig. 1). The controls identified nothing on the blots. It has been shown previously that MAG from developing rats and myelin-deficient *quaking* mice has a molecular weight larger (but only by 10,000 to 25,000) than MAG isolated from normal adult animals (refs 13, 14). Therefore, we examined the molecular weights of proteins with which the anti-MAG antibodies react in the developing human central nervous system (CNS) and in the brain stem: for example, at 31 weeks of

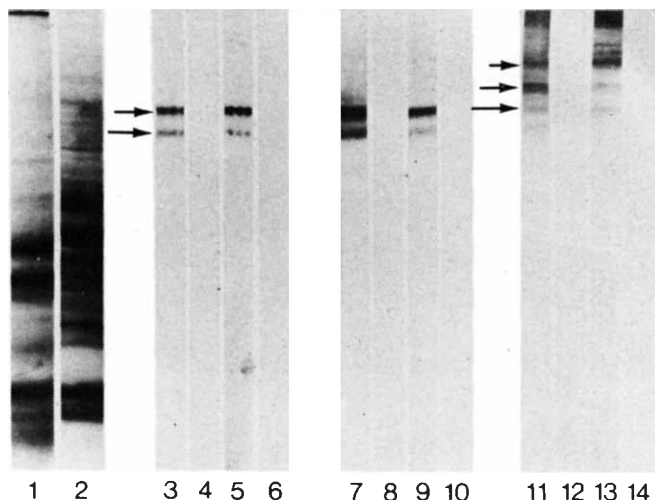


Fig. 1 Western blots of isolated MAG (lanes 3–6), homogenate of adult human spinal cord (lanes 7–10) and homogenate of human retina (lanes 11–14). The isolated MAG, the spinal cord homogenate and the retinal homogenate were run side by side on a 3-well SDS-polyacrylamide gel using the Fairbanks system¹⁹ (lane 1 is a Coomassie blue-stained gel of the spinal cord homogenate and lane 2 is the same for retinal homogenate). The antigens were subsequently transferred electrically to nitrocellulose sheets. The nitrocellulose sheet was then cut into strips and the rest of the staining procedure was carried out with each of the strips in a capped test tube (for experimental details see ref. 9). Next, gel lanes 3, 7 and 11 were incubated with BRIC₃₇; 4, 8 and 12 with control monoclonal antibody; 5, 9 and 13 were incubated with the patient's (M) serum; and 6, 10 and 14 with a control serum from a patient with IgM monoclonal gammopathy without accompanying neuropathy. Then strips 3, 4, 7, 8, 11 and 12 were incubated with peroxidase-labelled rabbit anti-rat immunoglobulins and strips 5, 6, 9, 10, 13 and 14 were incubated with peroxidase-labelled rabbit anti-human IgM. The bound immunoglobulins were revealed by dipping the strips into diaminobenzidine- H_2O_2 solution. BRIC₃₇ (lane 3) and IgM from the patient's serum (lane 5) bound to isolated MAG (short arrow) and dMAG (long arrow) (dMAG is a degradation product of MAG²⁰) and only to MAG and dMAG in the spinal cord homogenate (lanes 7 and 9). In the retinal homogenate both BRIC₃₇ (lane 11) and IgM from the patient's (M) serum (lane 13) identified three major bands: one with a relative mobility identical to MAG (longest arrow), another with a M_r ~130,000 (middle arrow) and the third with a M_r of ~200,000 (shortest arrow). The control monoclonal antibody (lanes 4, 8, 12) and the control serum (lanes 6, 10, 14) were totally negative. There are some differences between BRIC₃₇ and IgM from the patient's (M) serum with respect to relative intensity of the bands. BRIC₃₇, for example, stains dMAG in the spinal cord homogenate (lane 7) more intensely than does IgM from the patient's serum (lane 9). BRIC₃₇ also brings out dMAG in the retinal homogenate (11) whereas IgM from the patient's (M) serum does not (13). However, IgM from the patient's (M) serum stains the 200,000- M_r protein in the retinal homogenate (13) more darkly than does BRIC₃₇ (11). These differences are probably a reflection of the fact that BRIC₃₇ and the IgM from the patient's (M) serum do not react with the same epitopes on MAG. BRIC₃₈, which appears to bind to the same epitope as BRIC₃₇, gives the same relative intensities of bands on blots of retina and spinal cord as does BRIC₃₇.

gestation the antibodies react with only two pairs of bands, one of M_r >250,000, the other of M_r ~200,000. At 37 weeks of gestation the anti-MAG antibodies react with three bands: one of M_r ~225,000, another of ~200,000 and the third one ~100,000. At 7 months postnatal the anti-MAG antibodies react only with an antigen having a molecular weight of 100,000 (L.S.M. and K.S., in preparation). It seems, therefore, that the 'immature' MAG in the human CNS is larger than that in rodents. All the bands identified by the anti-MAG antibodies in the human retina, including the 130,000- M_r protein, are

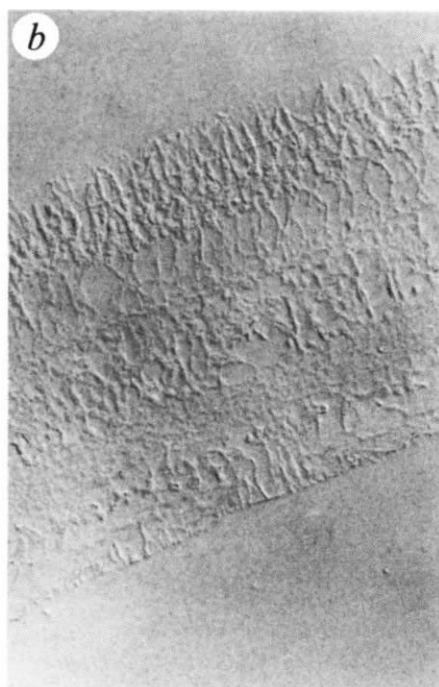
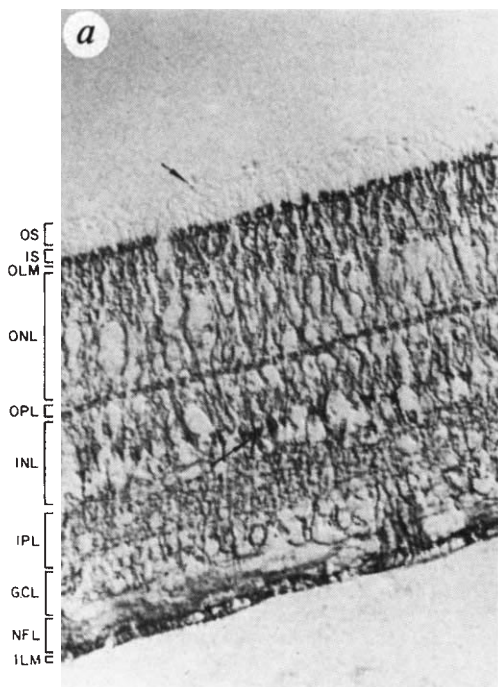


Fig. 2 *a*, Section of formalin-fixed, paraffin-embedded human retina stained with BRIC₃₇ (PAP technique). The photograph is taken through differential interference optics (Nomarski), $\times 400$ before photographic conversion. There is intense staining of processes throughout the retina. The long arrow points to the location of nuclei of Müller cells, the short arrow to unstained photoreceptors. OS, outer segment of photoreceptors; IS, inner segment of photoreceptors; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fibre layer; ILM, inner limiting membrane. *b*, A section from the same block as that shown in *a*, stained with one of the controls and photographed in the same manner as *a*.

identified in the human CNS at one time point or another during development. Thus, it appears that the antigens in the retina with which the anti-MAG antibodies react may be a mixture of the 'immature' and 'mature' forms of MAG.

The normal human retina is unmyelinated throughout life¹ yet the retina is the only site at which MAG has been found outside the myelin sheath and myelin-forming cells—this observation underlines the need to look beyond myelination in

the search for a functional role for MAG. It is also of interest that in the retina MAG appears to be present in Müller cells. Barnstable¹² has described a monoclonal antibody which in the rat retina reacts only with Müller cells and also binds to astrocytes in all regions of the brain. There are several other features that astrocytes and Müller cells have in common, such as close apposition to neuronal processes¹⁵, potassium ion transport¹⁶ and γ -aminobutyric acid transport¹⁷. However, it has been demonstrated that in the nerve fibre layer of chicken retina, Müller cells form a myelin-like sheath around thick axons¹⁸. Therefore it could be argued that they bear some resemblance to oligodendrocytes and Schwann cells. The presence of MAG on human Müller cells adds a new feature to that resemblance and provides another reason for considering the possibility that the Müller cells may be more than simply specialized retinal astrocytes.

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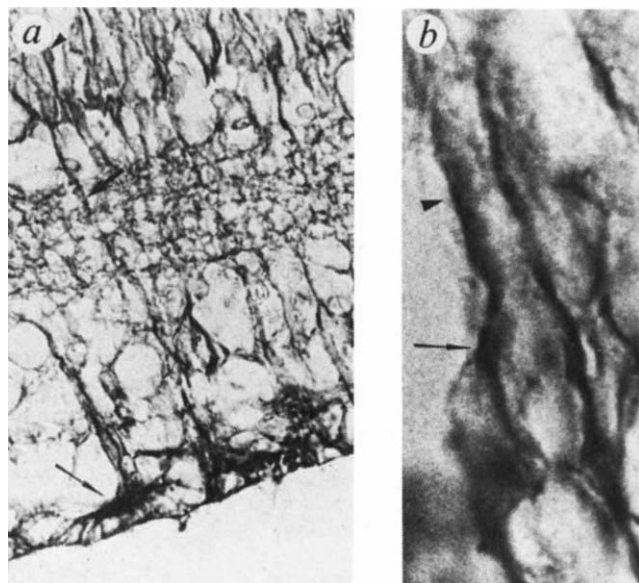


Fig. 3 *a*, Section of paraffin-embedded human retina stained with BRIC₃₇ (PAP technique). The long arrow points to the footplate of a Müller cell; the arrowhead to a perinuclear area of a Müller cell which is shown at higher magnification in *b*. A process from this cell can be followed all the way to the inner limiting membrane (thick arrow) (Nomarski $\times 300$). *b*, The perikaryal area of a Müller cell (arrow) and a characteristically broad ascending process (arrowhead). Only the plasma membrane is stained. There is no staining of the nucleus or the cytoplasm (Nomarski $\times 1,200$, oil immersion).

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Modulation of the response of a photosensitive muscle by β -adrenergic regulation of cyclic AMP levels

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The light-induced constriction of the irises of some vertebrates is mediated by photosensitive pupillary sphincter cells, which have rhodopsin molecules in their sarcolemmas¹⁻⁶. Light-induced isomerization of these rhodopsin molecules leads to the release of Ca^{2+} from an internal pool, which in turn activate the contractile proteins^{2,4,7}. A central nervous reflex is therefore not essential for the light responsiveness of these irises, but they do appear to be innervated⁶⁻¹⁴. The photosensitive iris of the toad receives sympathetic (adrenergic) innervation. Stimulation of sympathetic nerves to the eye¹¹ or application of adrenergic agonists to the iris^{8,14} cause pupillary dilation due to relaxation of the sphincter muscle. We show here that β -adrenergic stimulation of toad sphincter cells modulates their photoresponses by elevating the intracellular levels of cyclic AMP. However, cyclic AMP does not appear to be involved in the transduction event but rather alters the availability of Ca^{2+} for contraction.

Irises of the toad *Bufo marinus* were isolated by opening the eye cup posteriorly and removing the lens and sclera, except for a small circumferential strip of sclera attached to the iris. This scleral strip was pinned with the anterior iridial surface upwards to a clear silicone rubber substrate in a perfusion chamber and placed in an IR pupillometer¹³. The output of the pupillometer was calibrated as pupillary area (mm^2) and provided continuous monitoring of iridial strip responses to both light and drug treatment. The scleral strip and attached iris were pinned so as to avoid any external tension on the sphincter muscle. The toad iris does possess a dilator muscle (contrary to the results of Armstrong and Bell⁸) but it is unresponsive to either light or adrenergic stimulation¹⁵.

Addition of adrenergic agonists to irises causes a pronounced pupillary dilation and variable changes in the size of light responses. This response is elicited most easily by the β -adrenergic agonist isoprenaline and least easily by the α -agonist phenylephrine, suggesting that the effect of adrenergic stimulation is mediated by β -adrenergic receptors. This is supported by the observation: (1) the effect of isoprenaline, adrenalin or phenylephrine is blocked by the β -antagonist propranolol (Fig. 1), but not by the α -antagonists phentolamine or phenoxybenzamine; (2) the response is elicited by a 1,000-fold lower concentration of isoprenaline than phenylephrine; and (3) propranolol antagonizes the pupillary response elicited by high-potassium depolarization and subsequent release of endogenous neurotransmitter¹⁵. Low concentrations of isoprenaline (10^{-9} – 10^{-8} M) always cause pupillary dilation of dark-adapted, previously untreated irises ($n = 23$). However, the sensitivity of the light response to isoprenaline treatment varies slightly between animals. Low concentrations of isoprenaline affect light responses by either increasing ($n = 16$) or decreasing ($n = 7$) the peak-to-peak amplitude of responses. In cases where isoprenaline causes an increase in the amplitude of light responses, higher concentrations of isoprenaline (5–10 times) cause a decrease in response amplitude. However, in most cases the decrease of response amplitude caused by isoprenaline is transient (Fig. 2). One possible explanation is that the β -adrenergic receptors desensitize rapidly. However, since this

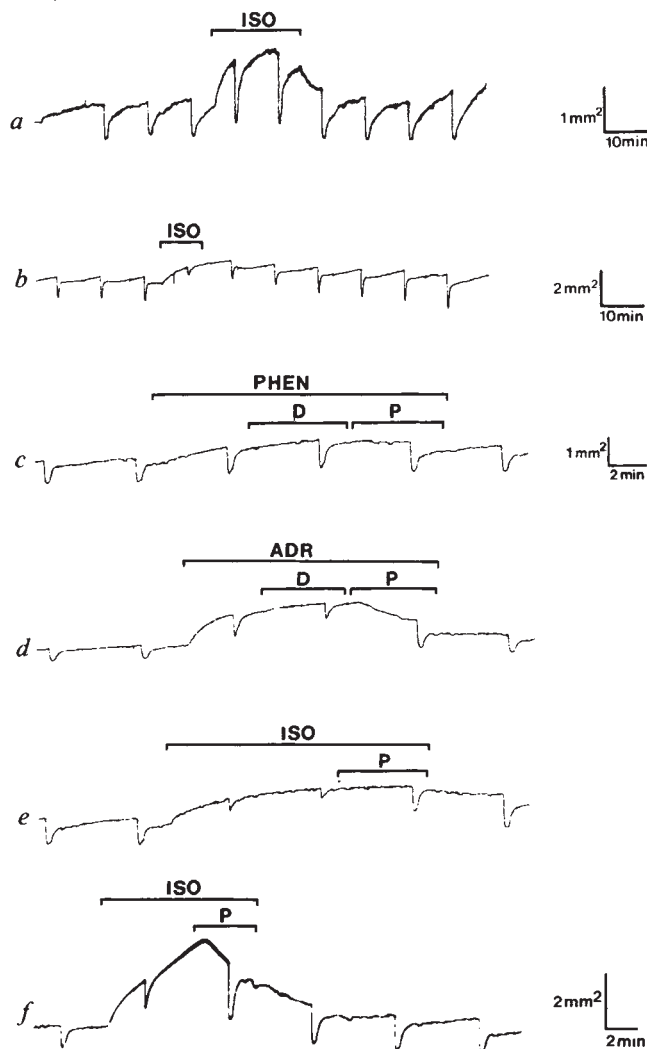


Fig. 1 Tracings of the output of the pupillometer (pupillary area). Stimulation with light causes a downward deflection of the tracing and indicates a decrease in pupillary area equivalent to pupillary constriction. Effect of the adrenergic agonist isoprenaline on pupillary size and light response in four different irises. *a, b*, Addition of isoprenaline (ISO, 10^{-8} M) caused pupillary dilation in both cases. However, in one case isoprenaline increased the peak-to-peak amplitude of light responses (*a*) and in the other case it decreased the amplitude of light responses (*b*). *c-e*, Tracings from the same preparation. The iris dilates in response to addition of phenylephrine (PHEN, 10^{-6} M), adrenaline (ADR, 10^{-6} M) or isoprenaline (ISO, 10^{-7} M) to the perfusate. Effect of phenylephrine and adrenaline is not blocked by the α -blocker phenoxybenzamine (D, 10^{-6} M) but is reversed by the β -antagonist, propranolol (P, 10^{-6} M), although the reversing effect on phenylephrine response in this iris is slower. Propranolol (P, 10^{-6} M) blocks the effect of isoprenaline (10^{-7} M) on light responses but does not rapidly return the dark-adapted iris to pretreated levels of dilation. Concentrations of propranolol 100 times the isoprenaline concentration do block and reverse the effect of isoprenaline (*f*). All drug solutions are prepared fresh from frozen stock solutions (10^{-3} M dissolved in 2–5 mM HEPES Ringer's, pH 4.8). Stock solutions of agonists are supplemented with ascorbic acid (50 mg l^{-1}) to retard oxidative degradation²⁹. Perfusion rate was one to four times the chamber volume per min. Light stimulus intensity was 16.45 mW cm^{-2} .

effect is not transient (up to 1 h) if a cyclic AMP-phosphodiesterase (PDE) inhibitor is also present, a more likely explanation is an increase in PDE activity and subsequent reduction of cyclic AMP levels.

The toad iris appears to resemble other systems in that β -adrenergic receptors affect cells by activating adenylate cyclase

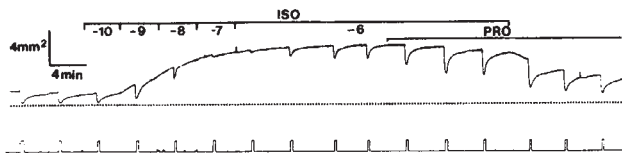


Fig. 2 Effect of progressively higher doses (log concentrations) of isoprenaline (ISO) on pupillary responses to light. Lower trace is the output of a light monitor. Dotted line is an arbitrary reference line. Low concentrations of isoprenaline (10^{-9} M) cause pupillary dilation and slightly increase the amplitude of the light response. To decrease the amplitude of light responses the concentration of isoprenaline was increased successively. High concentrations of isoprenaline (10^{-6} M) still only transiently reduce the size of light responses. Addition of propranolol (PRO, 10^{-6} M) to an isoprenaline-treated iris partially antagonizes the effect of isoprenaline.

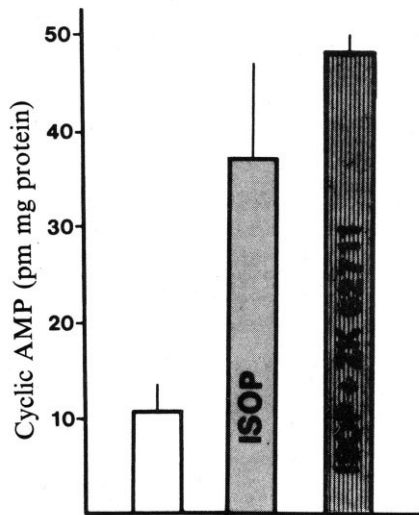


Fig. 3 Cyclic AMP levels of dark-adapted toad sphincters. The irises of 24 toads (*Bufo marinus*) were dissected in dim room light, pinned to a clear silicone rubber base in a Petri dish and then dark-adapted for 15–20 min while maintained in Ringer's solution. Because of the relatively small amount of tissue available for these measurements the largest toads (usually female) were used. In addition, each measurement was made from a pooled sample of four irises from four different animals, thus minimizing individual animal variations. Values represent the mean and standard deviation of the mean of untreated ($n=6$), isoprenaline-treated (ISOP, $n=3$, 10^{-7} M) and ZK62711 with isoprenaline-treated ($n=3$, 10^{-6} M) sphincters. After dark-adaptation the sphincter region was dissected from the remainder of the iris using IR viewers (FJW, Find-R-Scope) mounted on a dissection microscope. For drug treatment, tissue was then immersed in Ringer's solution with drug for 30–60 s and then frozen in liquid Freon at liquid nitrogen temperature. Untreated tissue was placed in fresh Ringer's for 30–60 s and then frozen. Frozen samples were homogenized in cold 5% trichloroacetic acid, centrifuged and the cyclic nucleotide content of the supernatant was determined according to the radioimmunoassay technique of Harper and Brooker¹⁷. The protein precipitate was processed according to the method of Lowry *et al.*³⁰.

and thereby increasing cellular concentrations of cyclic AMP¹⁶. Measurements of cyclic nucleotide levels by radioimmunoassay¹⁷ indicate that isoprenaline causes a fourfold increase in cyclic AMP levels (Fig. 3). Therefore, the pupillary dilation and decrease in the size of light responses observed with isoprenaline treatment may be due to an increase in cyclic AMP levels. This is supported by the enhancement of the effect of isoprenaline on light responses by other agents which increase cyclic AMP levels. Treating irises with PDE inhibitors such as isobutyl methylxanthine (IBMX, $n=3$) or the cyclic AMP-specific PDE inhibitors ZK62711 ($n=10$, Shering)^{18,19} or RO201724 ($n=3$, Hoffman LaRoche)^{18–20} but not the cyclic GMP-specific PDE inhibitor MB22948 ($n=5$, May and Baker)^{18–21} causes a decrease in the size of light responses

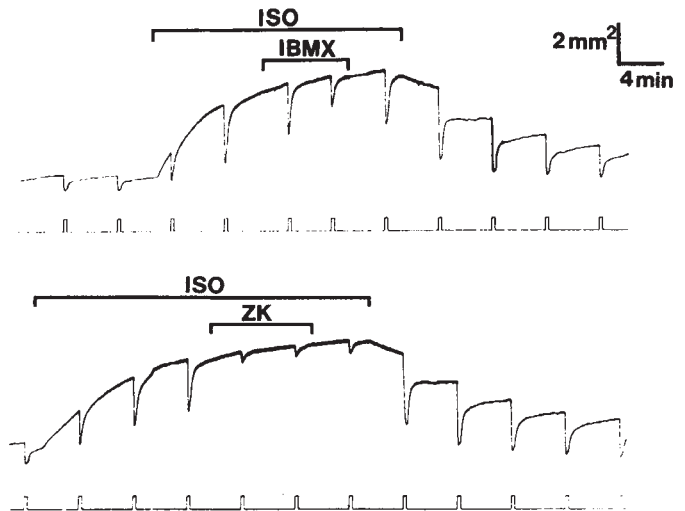


Fig. 4 Potentiating effect of the phosphodiesterase inhibitors ZK62711 and isobutyl methylxanthine (IBMX) on the response of the iris to isoprenaline. Isoprenaline (ISO, 5×10^{-8} M) added to the perfusate bathing the iris caused pupillary dilation and increased the peak-to-peak amplitude of light responses. Treating the iris with IBMX (10^{-3} M) in the presence of isoprenaline reduces the size of light responses. ZK62711 (ZK, 10^{-6} M) in the presence of isoprenaline produced an even greater decrease in light responses which persisted for as long as the inhibitor was present with isoprenaline. ZK62711 alone had no effect on either the dark-adapted iris or on light responses.

beyond that caused by isoprenaline alone (Fig. 4). Irises treated with isoprenaline and ZK62711 contain slightly higher levels of cyclic AMP than irises treated with isoprenaline alone (Fig. 3). In addition, the effect of isoprenaline on light responses is potentiated by cholera toxin ($n=2$). Cholera toxin is known to enter cells and activate adenylate cyclase irreversibly thereby increasing the intracellular concentration of cyclic AMP^{22,23}. Potentiation of the effect of isoprenaline on iridial light responses by specific cyclic AMP-PDE inhibitors and by cholera toxin suggests that cyclic AMP is involved in an inhibition of light-induced contractions.

Although cyclic AMP appears to modify the iridial response to light, preliminary results of another study indicate that cyclic nucleotide levels are not regulated by light in this particular photoreceptor. Furthermore, in the absence of stimulation of β -adrenergic receptors, PDE inhibitors have little or no effect on either the size of the dark-adapted iris or on light responses, suggesting that the basal activity levels of the cyclase as well as the PDE are low. Thus it seems unlikely that activation of a PDE^{24,25} and subsequent lowering of cyclic nucleotide levels are involved in phototransduction in the sphincter muscle. The most likely explanation is that cyclic AMP exerts its effect on light responses after the initial transduction events.

There are at least two places where cyclic AMP can act to modulate a contractile response in smooth muscle. Cyclic AMP can inhibit the contractile response by initiating the phosphorylation of myosin light-chain kinase, thus preventing the activation of myofilaments²⁶. That is, Ca^{2+} released as a result of exposure to light would have no effect on inactivated myoproteins. Cyclic AMP also causes sequestration of Ca^{2+} by the sarcoplasmic reticulum, thereby making fewer Ca^{2+} available for contraction²⁷. Either or both of these mechanisms could be responsible for the effects observed in the present case. However, several pieces of evidence suggest that increased sequestration of Ca^{2+} rather than prevention of activation of myofilaments is the primary cause of the decrease in size of light responses. First, irises that are maximally dilated by agents such as isoprenaline still respond to light. This suggests that the myoproteins are functional. Second, normal irises respond to sustained illumination with a prolonged contraction, but isoprenaline-treated irises

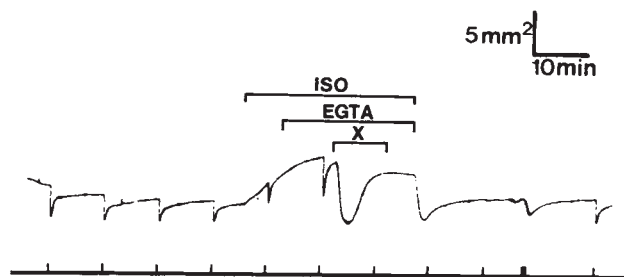


Fig. 5 Effect of calcium ionophore X-537A on the pupillary dilation caused by isoprenaline. In this experiment the iris was treated first with isoprenaline (ISO, 10^{-7} M) in normal Ringer's solution and then by isoprenaline in a low calcium, EGTA (EGTA, 10^{-4} M) solution before addition of the ionophore (X, 5×10^{-6} M) to the same solution. Further experiments indicated that the iris responds to isoprenaline treatment even in the absence of calcium in the perfusate. Therefore, in several experiments the calcium free-EGTA perfusate was started before the application of isoprenaline and ionophore. The results are the same as presented here. The ionophore X-537A causes maximum pupillary constriction and eliminates the response to light.

respond with only a transient contraction, as though Ca^{2+} released by light is rapidly resealed. Third, the light-induced contraction is larger after either the removal of isoprenaline or the addition of propranolol to an isoprenaline-treated iris. These findings suggest that calcium pools had been loaded during isoprenaline treatment and the stimulus for Ca^{2+} sequestering was then removed.

If the effect of elevated cyclic AMP levels on the sphincter is to increase the sequestering of Ca^{2+} and not affect the contractile proteins directly, then release of Ca^{2+} from these pools should cause a maximum contraction, even in the presence of elevated cyclic AMP levels. This appears to be the case, because perfusion of an isoprenaline-treated iris with the calcium ionophore X-537A²⁸ in a calcium-free EGTA Ringer's solution causes a rapid maximal pupillary constriction, followed by dilation and complete loss of the light response ($n=5$, Fig. 5). These results suggest that the myofilaments are capable of contracting during treatment with isoprenaline, but that Ca^{2+} is unavailable due to increased sequestration. The increased size of the light response immediately after some of the treatments with isoprenaline and the temporary absence of light responses after treatment with the ionophore may indicate that at least some of the Ca^{2+} stores augmented by cyclic AMP are sensitive to light.

These results indicate that the autonomous light response of the pupillary sphincter is modified by stimulation of β -adrenergic receptors. Thus, sympathetic innervation of the sphincter appears to regulate at least the amplitude of light responses by regulating the availability of Ca^{2+} for light-induced release. The most likely mechanism for calcium regulation in this system is a cyclic AMP-mediated sequestration of Ca^{2+} into stores; some of these stores are light sensitive. These results also imply that cyclic nucleotides are not directly involved in the transduction process of this muscle.

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Complementation in monocyte hybrids revealing genetic heterogeneity in chronic granulomatous disease

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Chronic granulomatous disease (CGD) is a rare syndrome, found predominantly in male children and characterized by life-threatening, recurrent infections. The superoxide (O_2^-)/hydrogen peroxide (H_2O_2) generating system in the granulocytes and monocytes of CGD patients is completely defective¹. Furthermore, a novel type of cytochrome *b*, detected by the optical spectrum of phagocytes from healthy subjects^{2,3}, is lacking in those of most male CGD patients⁴. In female CGD patients, the cytochrome *b* is present⁴, but cannot, as in normal cells⁵, be reduced on metabolic stimulation of the phagocytes in anaerobic conditions. Here, to demonstrate the importance of cytochrome *b* in this system and to investigate the genetic background of the various forms of CGD, we have hybridized monocytes from a cytochrome *b* negative, X-linked male CGD patient with monocytes from a cytochrome *b* positive, male CGD patient with unknown genetic background. Monocytes were used because they are the only blood phagocytes that show an active protein synthesis, whereas fibroblasts or lymphocytes do not express the $\text{O}_2^-/\text{H}_2\text{O}_2$ generating system. The heterologous hybrids were positive in the nitroblue tetrazolium (NBT) slide test, indicating the complementation of the $\text{O}_2^-/\text{H}_2\text{O}_2$ generating system, whereas the homologous hybrids remained negative, as did the non-fused cells of these patients. We thus conclude that cytochrome *b* is part of the $\text{O}_2^-/\text{H}_2\text{O}_2$ generating system and that somatic cell hybridization experiments with monocytes provide a means of studying the genetic background of CGD patients. We believe this to be the first report of genetic complementation by somatic cell hybridization experiments using monocytes instead of fibroblasts.

Because two patterns of inheritance of CGD seem to exist—one X-linked⁶ and one autosomal recessive⁷—Segal and co-workers³ have suggested that the absence of cytochrome *b* is correlated with the X-linked form of CGD and that the malfunction of cytochrome *b* is correlated with the autosomal recessive form. However, segregation between the expression of cytochrome *b* and the X-linked form of CGD has recently been described⁸. Moreover, we have discovered three cytochrome *b* negative patients (one male and two females) within one family with the autosomal recessive form of CGD, as well as one cytochrome *b* negative female CGD patient with an unknown genetic background. Table 1 shows the genetic heterogeneity of 12 male and 5 female CGD patients. The CGD families are unrelated to each other in the last four generations. It is clear

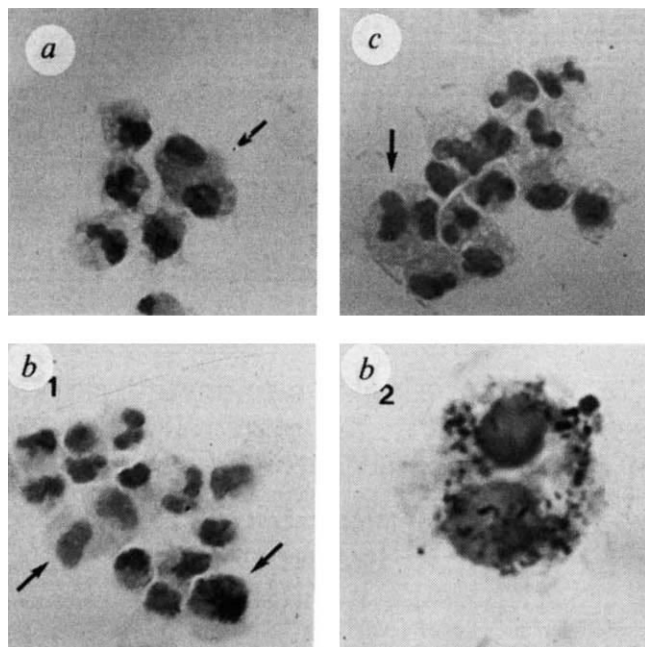


Fig. 1 NBT slide test on somatic cell hybrids of monocytes from two different CGD patients. Patient 1: X-linked, cytochrome *b* negative male; patient 2: cytochrome *b* positive male, mode of disease inheritance unknown. *a*, Fusion product of monocytes from patient 1 (negative in NBT test); *b*₁, fusion products of monocytes from patient 1 and patient 2 (one negative and one positive in NBT test); *b*₂, NBT-positive heterologous hybrid (higher magnification); *c*, fusion product of monocytes from patient 2 (negative in NBT test).

Methods: Monocytes (90–95% monocytes, 5–10% lymphocytes, 0–2% basophils) were isolated from mononuclear leukocytes by elutriation¹⁶, 3×10^7 cells were fused with polyethylene glycol (58%, molecular weight 4,000) for 1 min in Dulbecco medium¹⁷ and kept in maintenance culture in round-bottom wells of a microtitre plate in Iscove's modified medium with 10% fetal calf serum. Cells were collected at the indicated times and resuspended in medium of pH 7.4 containing 138 mM NaCl, 2.9 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.6 mM CaCl_2 , 1.0 mM MgCl_2 , 5.5 mM glucose and 0.5% (w/v) human albumin. Nitroblue tetrazolium (Sigma) was added at 0.5 mg ml^{-1} , and the cells were stimulated for 30 min with 100 ng ml^{-1} phorbol myristate acetate (Consolidated Midland Corporation). Thereafter, the cells were fixed in suspension with paraformaldehyde (1% v/v), spun onto a glass slide with a Shandon-Elliott Cytospin centrifuge (10 min at 600 r.p.m.), and stained with nuclear fast red (Merck Darmstadt) for 20 min for counting of the NBT-positive hybrids. Part of the unstimulated cells were stained with May-Grünwald (5 min) Giemsa (1 h) to count the total number of monocyte hybrids.

from Table 1 that considerable heterogeneity exists in CGD: 5 of the 12 male patients do not show the X-linked inheritance (compared with 4 out of 20 patients in ref. 4) and 3 (two sibs) of the 5 female patients were cytochrome *b* negative (compared with 2 cousins with partial cytochrome *b* deficiency out of 7 female patients in ref. 3). Furthermore, the discovery of the family with the autosomal recessive trait and absence of cytochrome *b* in their neutrophils, as well as the X-linked CGD patient with cytochrome *b* in this study and the one reported recently⁸, preclude correlation between the absence of cytochrome *b* and the X-linked form of CGD. However, the correlation between the absence of cytochrome *b* and CGD in general still holds. The one male patient without cytochrome *b* in 'unknown' category (Table 1) is comparable with the three male patients in ref. 3 without any affected relatives. Because the disease is often fatal in early childhood, a high incidence of spontaneous mutations in the X-linked form may be expected. This might explain why some male patients without cytochrome *b* have mothers and sisters without any sign of heterozygosity. The one female patient without cytochrome *b* in the 'unknown'

category is more difficult to explain. She might belong to the autosomal recessive form of CGD without cytochrome *b*, or to the X-linked form of CGD. In the latter instance, she would be heterozygous, with an extreme lyonization pattern (as yet undetected), while her mother would be heterozygous with an extreme lyonization pattern in the opposite way.

Whatever the mode of inheritance in these patients, Table 1 clearly shows that the expression of the $\text{O}_2^-/\text{H}_2\text{O}_2$ generating system is controlled by at least three genetic loci, one or more of which is located on the short arm of the X chromosome^{9,10}.

As long as the molecular defects remain unknown, the only way to study the genetic background of the various CGD patients is by complementation analysis of somatic cell hybrids. Generally, fibroblasts are used for this purpose; however, these cells do not express the $\text{O}_2^-/\text{H}_2\text{O}_2$ generating system, as measured with the NBT reduction test (ref. 11, confirmed by us). Lymphocytes have also been reported to express the $\text{O}_2^-/\text{H}_2\text{O}_2$ generating system¹², but these cells, too, failed to reduce NBT in our hands (unpublished). The only way to circumvent this problem is to perform complementation studies with bone marrow cells or monocytes, because these cells display an active protein synthesis. For obvious reasons, the use of bone marrow cells is excluded, leaving the possibility of monocytes. However, this implies that very few hybridization experiments can be performed, because for one experiment 100 ml of fresh blood from two patients are required. Cryopreserved monocytes cannot be used, because in control experiments we found that the latter cells yielded somatic hybrids that quickly deteriorate in culture.

The large blood sample is needed for the following reasons: (1) the fusion efficiency of monocytes is 0.4–1%, and at least 2×10^7 monocytes per fusion are required for a good recovery of viable hybrids; (2) the monocytes must be highly purified (>90%) to avoid fusion between monocytes and contaminating cells, which would lead to false-negative results; (3) to meet the above-described criteria and to perform two homologous and one heterologous fusion experiment, 3×10^7 purified monocytes are needed, equivalent with 100 ml of whole blood.

To establish the different complementation groups, multiple blood samples from some patients may be needed. This would pose problems because most patients are small children. Therefore, we decided to try the most obvious complementation experiment, fusion of monocytes from an X-linked, cytochrome *b* negative male (patient 1) with those of a cytochrome *b* positive male (patients 2 and 3), to show not only the feasibility of monocyte hybridization but also the importance of cytochrome *b* for the activity of the $\text{O}_2^-/\text{H}_2\text{O}_2$ generating system. The results of these experiments are shown in Fig. 1 and Table 2.

Figure 1 clearly shows that NBT-positive hybrids were only found in the heterologous situation (expt *b*). The number of positive hybrids observed directly (0 h, Table 2, expt *d*) or shortly after (2.5 h, Table 2, expt *b*) fusion was already 30% of all hybrids, indicating that protein synthesis was not required for complementation in these particular experiments. Indeed, in an experiment parallel to expt *d* (Table 2), inhibition of protein synthesis with cycloheximide ($1 \mu\text{g ml}^{-1}$) had no effect on the percentage of positive hybrids up to 40 h (not shown). This finding, however, does not preclude the use of monocytes, because protein synthesis might be needed in other complementation groups. The decline in the number of positive hybrids after 40 h in expt *b* is probably due to deterioration of the hybrids. The percentage of positive hybrids at 15 h is very close to the expected maximal value of 50%, indicating that complementation only occurs when heterologous hybrids are formed. The homologous hybrids remained NBT negative, as did the non-fused cells of both patients. In separate experiments, we found that all monocyte hybrids from a single normal donor remained NBT positive, as did non-fused normal cells. Thus, we conclude that both types of CGD patient are genetically different, and that cytochrome *b* is most probably one of the corrective factors. Furthermore, monocyte hybridization experiments can provide information on the genetic background of

Table 1 Genetic heterogeneity in 17 patients with chronic granulomatous disease

	Mode of inheritance					
	X-Chromosomal cytochrome <i>b</i>		Autosomal recessive cytochrome <i>b</i>		Unknown cytochrome <i>b</i>	
	Positive	Negative	Positive	Negative	Positive	Negative
No. of male patients	(1)*	6	—	1	3	1
No. of female patients	—	—	—	2	2	1

CGD patients were diagnosed clinically by recurrent infections of skin, lungs and lymph nodes with catalase-positive bacteria and yeasts, and biochemically by failure of their blood granulocytes to mount a respiratory burst (O_2 consumption¹³, NBT reduction (see legend to Fig. 1), H_2O_2 formation¹⁴) on incubation with opsonized zymosan or phorbol myristate acetate, and reduced capacity of these cells to kill opsonized *Staphylococcus aureus in vitro*. X-chromosome linkage is concluded from the detection of heterozygosity in the mothers and sisters of the patients (subnormal values for O_2 consumption and H_2O_2 production, subnormal amount of cytochrome *b*, and mosaicism in the NBT slide test). Autosomal recessive inheritance is concluded from the following findings. In this family one male and two female CGD patients were found; a sister showed heterozygosity in the metabolic parameters and in the amount of cytochrome *b* (41% of normal), but no mosaicism in the NBT slide test; the father and the mother (first cousins) had low-normal metabolic activity in their neutrophils, no mosaicism in the NBT slide test and 59% and 64%, respectively, of the normal amount of cytochrome *b*. 'Unknown' comprises the category of GCD patients whose parents, brothers and sisters were found to be normal with respect to metabolic and functional tests. The presence of cytochrome *b* was evaluated from optical difference spectra of dithionite-reduced neutrophils minus native neutrophils; the amount of cytochrome *b* was calculated from these spectra by using an absorbance coefficient of $106 \text{ mM}^{-1} \text{ cm}^{-1}$ at 425 nm and of $21.7 \text{ mM}^{-1} \text{ cm}^{-1}$ at 558 nm (ref. 15).

* The amount of cytochrome *b* in the neutrophils from this patient was not measured accurately due to a slight contamination of the cells with haemoglobin; this resulted in a double peak at 425 nm and 433 nm in the redox spectrum; thus, a partial deficiency of cytochrome *b* may exist.

Table 2 Expression of the NBT reductase activity in somatic cell hybrids of monocytes from three CGD patients

Time after fusion (h)	% of NBT-positive hybrids in fusion experiment				
	<i>a</i> (1×1)	<i>b</i> (1×2)	<i>c</i> (2×2)	<i>d</i> (1×3)	<i>e</i> (3×3)
0	—	—	—	32.1 (<i>n</i> = 6)	0 (<i>n</i> = 3)
2.5	2.5 (<i>n</i> = 2)	30.1 (<i>n</i> = 4)	1.7 (<i>n</i> = 3)	—	—
15	0 (<i>n</i> = 2)	43.6 (<i>n</i> = 10)*	0 (<i>n</i> = 2)	45.1 (<i>n</i> = 3)†	0 (<i>n</i> = 4)
40	0 (<i>n</i> = 2)	22.6 (<i>n</i> = 7)*	0 (<i>n</i> = 2)	35.1 (<i>n</i> = 4)†	0 (<i>n</i> = 2)
136	0 (<i>n</i> = 1)	25.9 (<i>n</i> = 4)*	0 (<i>n</i> = 1)	—	—

The percentage of NBT-positive hybrids was calculated as the percentage of the number of hybrids counted per 5,000 cells per slide (4–40 hybrids); 1 to 10 slides were evaluated. Results are in geometric means derived from the log-normally distributed values. Expts *a*, *b* and *c* are as in Fig. 1; expts *d* and *e* were performed 1 month later with a second blood donation of patient 1.

* Student's *t*-tests performed on the log-normally distributed values, corrected for separate variance estimates, indicated two-sided *P* values of 0.08 for 2.5 h versus 15 h, 0.003 for 15 h versus 40 h, and 0.5 for 40 h versus 136 h.

† Student's *t*-tests performed as described above. *P* = 0.06 for 0 h versus 15 h, and 0.2 for 15 h versus 40 h.

CGD patients. We feel that only a multi-centre follow-up of this study will lead to the elucidation of a full set of complementation groups within reasonable time, because it is impossible to obtain these large quantities of blood frequently from the relatively small number of patients in one institute. The establishment of complementation groups may well lead to a better understanding of the genetic defects underlying CGD.

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Note added in proof: Fusion of monocytes from patients 2 and 3, and of monocytes from patient 1, with another X-linked patient did not result in complementation even after prolonged culturing.

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Amino acid sequence of rat submaxillary tonin reveals similarities to serine proteases

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Tonin¹, an esteroprotease isolated from rat submaxillary gland, is a serine protease with trypsin- and chymotrypsin-like activity. The substrate specificity of tonin shows that it differs from kallikreins and is definitely not a renin-like enzyme or an angiotensin-converting enzyme². Tonin can produce directly the vasoactive peptide angiotensin II, from angiotensin I, angiotensinogen and the synthetic tetradecapeptide substrate of renin by cleavage of a Phe-His bond. It has also been found to cleave some Phe and Arg bonds in various substrates such as β -lipotropin (β -LPH), adrenocorticotropin (ACTH), pro-

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opiomelanocortin (POMC)³ and substance P⁴. Here we describe the complete amino acid sequence of rat submaxillary gland, tonin. Comparison of the sequence of 219 amino acids with other serine proteases, particularly kallikreins, γ -subunit of nerve growth factor (NGF) and the recently described γ -renin, reveals extensive similarities. More interestingly, it also reveals the substitution of an Asp residue always found in the serine protease active site triad (Asp, His, Ser) by a Leu residue. This unusual substitution does not seem to affect the proteolytic activity of the enzyme.

Previous reports on the primary structure of rat tonin have shown that isoleucine and proline occupy the amino and carboxy termini respectively and that sequence analysis of 95 residues showed a very high homology to the serine protease family, especially to the γ -subunit of mouse NGF^{5,6}. The complete amino acid sequence of submaxillary gland tonin has now been determined (Fig. 1). Tonin is a single polypeptide chain of 219 amino acids including 10 cysteines and having a molecular weight of 23,796. This molecular weight is much lower than those previously reported which were all in the region of 28,000–30,000 and obtained experimentally by gel filtration, high pressure gel permeation, sedimentation equilibrium and gel electrophoresis experiments^{7,8}; this, in turn, led to an overestimation of the actual number of amino acids previously reported as 272⁵. The deduced sequence shown in Fig. 1 was obtained through analysis of the reduced and alkylated chain, of fragments derived from autolysis cleavages and from cyanogen bromide fragmentation. Details of the sequence, most of it done at the 10–20 nmol range, will be reported elsewhere (C.L. *et al.* in preparation).

Comparisons of tonin amino acid sequence with the published sequences of serine proteases (Table 1) show a very high homology (over 50%) between tonin and the various sequence of kallikrein including the pig pancreatic kallikrein⁹, the murine glandular kallikrein deduced from a cloned cDNA fragment¹⁰, the rat submaxillary kallikrein¹¹, which itself proved entirely homologous (117 out of 118 residues) to the rat pancreatic kallikrein deduced from the nucleotide sequence of the mRNA¹². Moreover, the tonin sequence is also very similar to the γ -subunit of murine NGF¹³ as proposed earlier⁶ and also to another enzyme found recently in the mouse submaxillary gland, γ -renin¹⁴. Interestingly, the γ -renin can produce angiotensin I from the renin substrate tetradecapeptide, but not from the natural renin substrate, whereas tonin can act on both substrates to produce angiotensin II. It is thus quite clear, as judged by Table 1, that tonin, NGF γ -subunit, γ -renin and probably epidermal growth factor (EGF) binding protein, form a closely related group within the kallikrein family. That relationship has already been documented by Bothwell *et al.*¹⁵ for glandular kallikrein and growth factor proteases of mouse submaxillary gland.

Looking at the actual sequence, it can be seen that the 10 half-cystine residues in tonin, when aligned to maximize homology can be found in similar positions in the NGF γ -subunit and in trypsin. From this alignment, the linkage of the disulphide bridges in tonin can thus be inferred so that Cys-7 is linked to Cys-131 (corresponding to trypsin 13 to 143), Cys-24 to Cys-40 (trypsin 31 to 47) which forms the 'histidine loop' responsible for proper orientation of the His residue in the active site, Cys-110 to Cys-177 (trypsin 115 to 216), Cys-142 to Cys-156 (trypsin 154 to 168) which forms the 'methionine loop', Cys-167 to Cys-192 (trypsin 179 to 203) which link together the primary and secondary binding sites. It can also be deduced that tonin, like the kallikreins, should not be expected to bind calcium ions since those residues in trypsin, namely Gln-58, Asn-60, Val-63 and Gln-68, are replaced in tonin by Arg-51, Asn-53, Lys-56 and Ala-61. It can also be deduced that tonin should not contain any carbohydrates, at least not the Asn-linked type, since the typical Asn-Xaa-Ser or Asn-Xaa-Thr recognition sequence cannot be found. This differs from pig pancreatic kallikrein where two glycosylation sites were found⁹, from the NGF γ -subunit where one site was found¹³ and possibly also from the rat

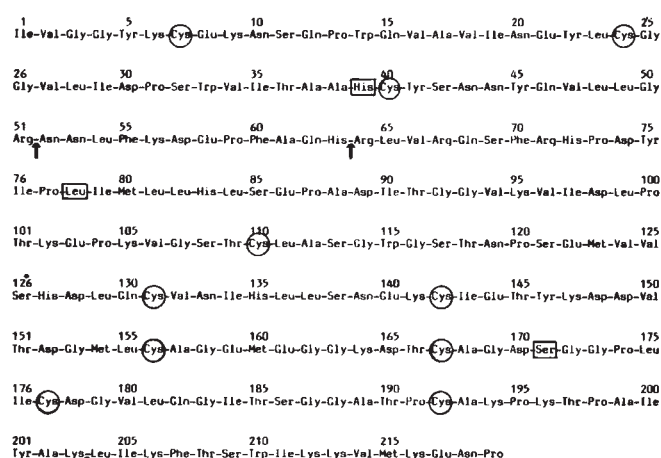


Fig. 1 Amino acid sequence of rat submaxillary gland, tonin. Cys residues are encircled and the residues localized at the active site are enclosed in boxes. Arrows indicate potential sites of autolysis. Positions 1–66 were obtained by sequencing the reduced and alkylated protein. Positions 64–86 were obtained by sequencing the autolysis fragment beginning at position 64. Positions 81–120 were given by the sequence of the cyanogen bromide-derived fragment starting at 81 and also of a fragment comprising residues 81–123. Positions 124–154 were obtained by sequencing the first 29 residues; the last two residues were localized as Gly-Met because position 154 is a CNBr cleavage site. Positions 155–200 and 161–200 were deduced by sequencing the respective cyanogen bromide fragments; positions 201–203 being deduced by amino acid composition. The last 15 residues were obtained by sequence of an autolysis fragment starting at position 204; the C-terminal Pro is deduced by amino acid composition and confirmed by previous studies⁵.

pancreatic kallikrein where a glycosylation recognition sequence can be seen (see note added in proof).

All but two of the 29 invariant residues of the serine protease family are conserved in the tonin sequence including the His and Ser residues found in the active site. Indeed, the sequence Val-Leu-Thr-Ala-Ala-His-Cys, found in every serine protease, is present; it does contain a conservative substitution, namely the isoleucine at position 35 in place of the common leucine residue. The complete sequence Cys-Xaa-Gly-Asp-Ser-Gly-Gly-Pro-Leu surrounding the active site Ser is also found in tonin at positions 167–175. One of the exceptions is at position 135 where an His residue replaces Pro. This particular position is characteristically variable in kallikrein and kallikrein-related enzymes as shown by the presence of an Asp in rat pancreatic kallikrein, a Thr in the pig pancreatic and mouse submaxillary kallikrein and a Lys in the γ -subunit of mouse NGF and in the γ -renin. The other exception, at position 78, is more interesting since it involves the replacement of the Asp residue localized at the active site (corresponding to Asp-102 of chymotrypsin) by a Leu residue; this change will be discussed later. The Asp-189 of trypsin, thought to determine the substrate specificity, is also present at position 165 in tonin thus explaining its trypsin-like character. The secondary binding site corresponding to the sequence Ser-Trp-Gly (trypsin position 214–216) is localized at position 186–188 where the Trp is replaced by a Gly. The Gly at position 216 is conserved in tonin (position 188) whereas the Gly at position 226 is replaced by an Ala in tonin (position 199). Both residues are located at the entrance of the binding cavity.

When aligning the sequence to maximize homology, it is necessary to introduce a two-residue deletion between position 20 and 21 with respect to all kallikreins and kallikrein-related enzymes. Another deletion of the same length is introduced between position 120 and 121 compared with pig kallikrein. One of the most striking features of the tonin sequence is the presence of a sizeable deletion (Fig. 2) between residue 73 and 74. To accommodate other kallikrein sequences, it is necessary

to introduce a 16-residue deletion which accounts for the shortened chain length of tonin corresponding to 219 amino acids instead of the 237 of rat pancreatic kallikrein and the 233 of the NGF γ -subunit. The only other serine protease having a comparable deletion is human plasmin (Fig. 2). Moreover, this region of the molecule is particularly sensitive to autolysis of the so-called 'kallikrein autolysis loop' leading to cleavages illustrated in Fig. 2. In the case of tonin, autolysis was not observed in that region even though three sites of cleavage, some of them probably due to autolysis were identified at positions 51–52, 63–64, and finally 203–204 (Fig. 1). The most often encountered being the one at 63–64, which could indicate a conformation different from the one found in kallikreins. Presence of autolysis sites seems to occur in all members of the kallikrein family and in some serine proteases. The presence of fragments arising from autolysis which complicates the sequence determination greatly, can be clearly seen upon reduction and alkylation of the disulphide bridges of tonin, of the γ -subunit¹⁶ and two serine proteases from the mouse submaxillary gland¹⁷.

An important striking feature of the tonin sequence is the absence of the Asp residue corresponding to Asp-102 (chymotrypsin numbering) present at the active site and its replacement by a Leu residue. This latter substitution is also accompanied by substitutions of the amino acids surrounding it (Fig. 2). This result was entirely unexpected since, in all serine proteases known so far, the Asp residue is thought to participate actively in the enzyme mechanism by forming what is known as the 'charge-relay system'. The existence of such a relay has been challenged, but examination of crystallographic data shows that the Asp residue is sufficiently close to the His to favour intramolecular interactions. On the other hand, some recent theoretical considerations by Genest and Ptak¹⁸ have shown that reinforced interactions between His and Ser, the latter being involved in the formation of the acyl-enzyme intermediate, must be accomplished with destabilization of the Asp-His diad. Also, Polgar and Bender¹⁹ have suggested that the Asp-102 does not take part in the charge-relay system but may act solely to stabilize the imidazolium intermediate. Moreover, the finding that papain, a thiol-protease containing His and Cys in the active site, reacts in a similar fashion to serine proteases could indicate that the Asp-102 might not be essential for proteolytic activity even though it might contribute to maintenance of a favourable conformation of the active site. On the other hand, it is clear that, even with such a substitution, tonin can act as a serine protease². With angiotensin I as substrate, the *pH* optimum of tonin was 6.8¹ whereas, with small synthetic substrates *pH* 8–9 was obtained² which is similar to values obtained with other serine proteases. It is inactivated by, for example,

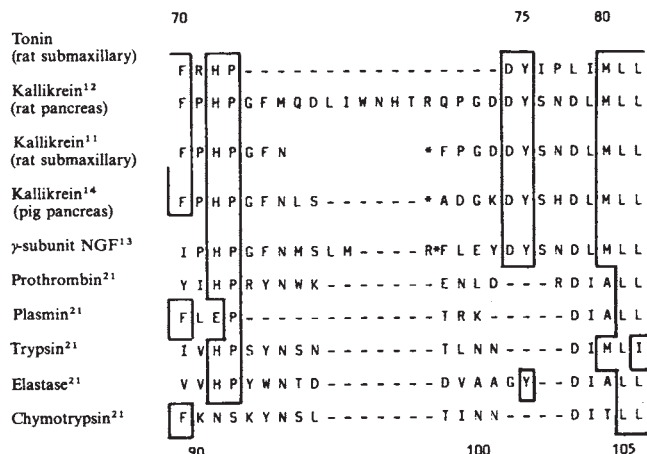


Fig. 2 Alignment of tonin 70–82 with corresponding segments of other serine proteases according to Dayhoff¹⁵ (in the single letter code²⁴). For ease of comparison, the numbering of chymotrypsin has been added below the alignment. The sign * in front of a sequence indicates the beginning of an observed autolytic fragment. Absence of dashes following a sequence indicates lack of sequence information.

diisopropylfluorophosphate (DFP) even though it requires much more DFP and longer incubation time for complete inactivation which could indicate modification around the active site as compared to other serine proteases. It is also able to cleave a certain number of Phe-Arg, Phe-Lys, Arg-Tyr, Arg-Trp, Phe-His, Phe-Phe and Phe-Gly bonds in various substrates. The only other substitutions known so far in active site residues concern the serine protease homologue, haptoglobin devoid of any proteolytic activity, where the triad His, Asp, Ser is replaced by Lys, Asp, Ala²⁰. Another explanation could be that the 16-residue deletion together with other amino acid substitutions could induce a conformational change sufficiently important as to bring close to the histidine and serine, another acidic residue not adjacent to Asp-102 in the sequence, but close in the three-dimensional structure. Even though intriguing, this explanation is difficult to reconcile with a deletion of similar size localized in the region of the plasmin enzyme that contains the normally encountered aspartic residue.

Final conclusions concerning both the importance of the deletion and of the substitution of Asp by Leu, will have to rely on current research on the three-dimensional structure of the enzyme.

Table 1 Homologies between serine proteases and rat submaxillary tonin

Enzymes	Species	No. of residues		Homology %	Chain length
		Compared	Identical		
Kallikrein ⁹ (submaxillary gland)	Rat	118	89	75.4	—
Kallikrein ¹⁰ (pancreatic)	Rat	237	170	71.1	237
Kallikrein ¹¹ (submaxillary gland)	Murine	149	97	65.1	—
γ -Subunit NGF ¹²	Murine	233	150	64.4	233
γ -Renin ¹³	Murine	73	46	63.0	—
Kallikrein ¹⁴ (pancreatic)	Porcine	232	119	51.2	232
Trypsin ²¹	Bovine	229	92	40.2	223
Plasmin ²¹	Human	237	83	35.0	229
Chymotrypsin ²¹	Bovine	240	76	31.7	231
Factor X ²¹	Human	241	67	27.8	—
Elastase ²¹	Porcine	245	68	27.8	240
Prothrombin ²¹	Human	262	71	27.1	—
Chymotrypsin ²²	Insect	234	61	26.1	216
Trypsin ²¹	Bacterial	237	61	25.7	221
Haptoglobin ²⁰ (β -chain)	Human	252	55	21.8	245
Factor B ²³	Human	288	46	16.0	—

The number of residues compared includes gap positions assigned to maximize homologies between the tonin sequence and the various proteases. Superscript numbers indicate the reference number.

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Note added in proof: Since submission of this manuscript work has clarified uncertainties at position 119-123. The results (included in Fig. 1) revealed the presence of an Asn-Pro-Ser-Glu-Met which could lead to N-glycosylation even though Bause and Legler²⁵ have shown that the Asn residue in such a sequence cannot be glycosylated because of the vicinal Pro.

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Computer-based characterization of epidermal growth factor precursor

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The cDNA sequence of the precursor of mouse epidermal growth factor (EGFP) has recently been reported by two groups^{1,2}, both of whom noted the presence of repeated similar segments, each about 40 residues long. One of these repeat units overlaps with the sequence of epidermal growth factor itself. The sequence of epidermal growth factor has been reported to be similar to that of pancreatic secretory trypsin inhibitor³ (PSTI) and a somewhat better match has been found with part of the sequence of bovine factor X⁴, one of the blood coagulating factors. We report here that there is an even stronger similarity between the sequences of some of the repeat units of epidermal growth factor precursor and certain segments in factor X. This sequence similarity is also apparent in comparisons with other blood coagulation factors. On the basis of these sequence comparisons we suggest a scheme for the evolution of the epidermal growth factor precursor. We have also identified certain structural features in the precursor sequence that bear on the way in which the mature epidermal growth factor is generated.

The EGFP precursor sequence was searched against 1,081 sequences (~120,000 residues) in the 1978 Dayhoff *Atlas*⁴ and about 800 sequences (~160,000 residues) in Newat⁵. The search

Table 1 Resemblances between homologous units in mouse EGFP and two adjacent segments of bovine factor X

EGFP Segment (residue)	Factor X _A (residues 44-84)			Factor X _B (residues 85-126)		
	% Identity	NAS	(s.d.)	% Identity	NAS	(s.d.)
a (22-64)	17.1	159	(0.2)	21.4	<u>238</u>	(2.7)
b (327-361)	34.3	314	(3.6)	37.1	<u>357</u>	(2.6)
c (362-402)	34.2	402	(4.8)	31.7	<u>463</u>	(10.5)
d (403-442)	22.5	288	(2.0)	32.5	<u>475</u>	(9.7)
e (443-484)	17.1	220	(0.4)	42.9	<u>488</u>	(10.2)
f (747-788)	29.3	354	(6.2)	35.7	<u>500</u>	(9.8)
g (838-876)	23.1	<u>359</u>	(4.1)	18.0	282	(1.5)
h (877-918)	31.7	<u>354</u>	(4.3)	26.2	286	(2.0)
i (919-960)	34.2	<u>402</u>	(6.2)	14.3	238	(1.3)
j (977-1,020)	31.7	<u>378</u>	(4.7)	19.1	238	(0.9)

Segments were compared by a computer program using a similarity measure based on the original algorithm of Needleman and Wunsch⁶. The normalized alignment score (NAS) reflects a penalty 2.5× the value of every match (10 points) (Cys matches, 20 points); it is adjusted for 100 residues. Scores over 150 usually indicate common ancestry⁵. The statistical likelihood of chance resemblance was empirically determined by comparing multiple jumbled sequences of the same lengths and compositions. If the alignment score of the genuine sequences is greater than 3.0 standard deviations (s.d.) above the mean of the jumble comparisons, common ancestry is generally inferred⁵. In contrasting A and B, the higher NAS is underlined in each case. Note that EGFP segments a-f are more closely related to factor X segment B, whereas g-j more closely resemble factor X segment A. EGFP segment j overlaps the known sequence for EGF itself.

identified bovine factors X and IX (in the Dayhoff *Atlas*), and human factors X and IX and bovine protein C (in Newat) as having matchable segments. In addition the search patterns revealed a repeat segment of 41±2 residues in the queried sequence, in good agreement with the size of the homologous segments reported by Gray *et al.*¹ and Scott *et al.*².

The nine segments from EGFP noted by Gray *et al.*¹ were designated b-j; a tenth homologue was denoted 'a' (Fig. 1). The two corresponding segments in the zymogen proteases were denoted A and B (Fig. 2). The sequences were compared by both similarity⁶ and difference^{7,8} types of computer alignment schemes. Each segment was compared with every other, and various phylogenetic trees were constructed⁹. Comparisons of the 10 homologous EGFP units with the factor X segments are given in Table 1. Interestingly, the units nearer the amino terminus more closely resemble segment B of factor X than they do segment A, whereas the inverse is true of the units nearer the carboxy terminus.

The similarities of EGFP units c-f to factor X segment B are such that the authentic alignment scores lie +10 standard deviations above the average of comparison sets made with jumbled sequences of the same lengths and compositions (Table 1). These similarities are of the same degree as lysozyme-lactalbumin or insulin-relaxin⁵ and are significantly better matches than the previously reported homology of EGF and PSTI (3) or, for that matter, EGF and factor X (4).

Several other homologies in EGFP were overlooked in the recent cDNA reports. There is a subtle but very significant homology between two large sections of the precursor sequence that are not in the cysteine-rich units (Fig. 1). The segment denoted x (residues 121-310) has a 30% identity with the segment denoted y (residues 561-746) (Fig. 1). It seems very likely that these two segments arose by internal duplication, a score of greater than +10 standard deviations being achieved when jumbled sequences of the same lengths and compositions were compared. In addition, there are four other shorter sequences that have similar sequences to each other; these are denoted q-t in Fig. 1.

There are a number of alternative explanations for the present structure of the EGF precursor. For example, it may have begun with a translocation of an 80-residue segment from the amino terminal region of factor X or a related zymogen. In this case the existence of the already duplicated segment (X_{AB}), would increase the chances of further mismatching and unequal crossing over. It is also possible that EGFP is the indirect result of

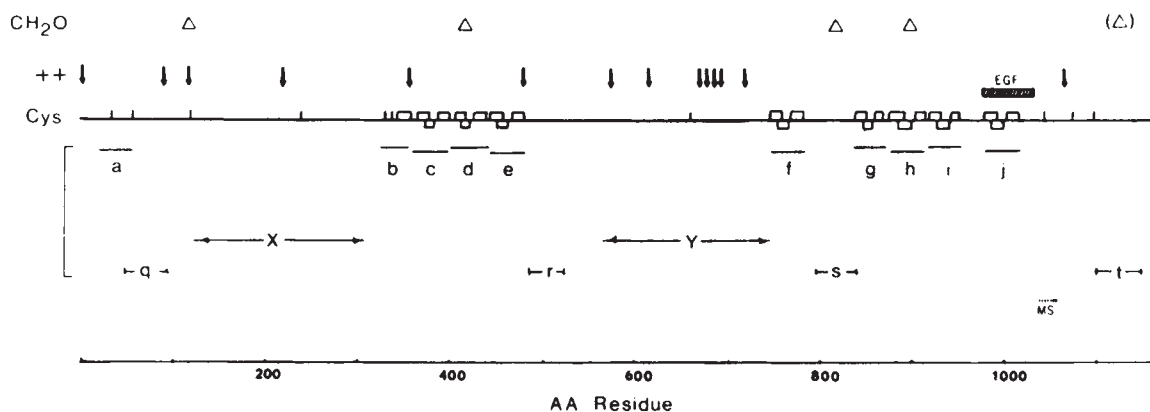


Fig. 1 Schematic depiction of mouse epidermal growth factor precursor (EGFP). The putative protein has 1,168 amino acids¹ (another report² suggests a sequence of 1,217 residues, but a comparison of the two cDNA sequences indicates that a difference of one base at position 3,751 puts the sequences out of register near the carboxy terminus). Three different kinds of homologous segments have been identified (*a-j*; *x-y*; *q-t*). These include nine previously noted homologous units designated *b-j*, and a tenth unit, less obvious, denoted *a*; EGF (residues 977-1,029) overlaps unit *j*. Two longer segments in EGFP are also homologous and are designated *x* and *y*. Finally, four other short segments are similar to each other and are denoted *q-t*. The disulphide bond arrangements in units *c-i* are patterned on the known arrangement in EGF (unit *j*). Other cysteines (Cys), including one in a predicted membrane-spanning segment (residues 1,039-1,059), have been left unpaired even though most are likely in the form of disulphide bonds. Triangles indicate consensus carbohydrate-attachment points; if the model described in the text is correct, only those situated outside the plasma membrane will be derivatized. Arrows denote 'double-basic' (Arg-Arg, Arg-Lys, and so on) potential proteolysis points.

	A	B	
Protein C-Bovine (res. 44 - 135)	YSDGDQcEDRPSGSPcDLPCcGR-GKcIDGLGGFRcDcAEGW--EGRFclHEVVRFSNcSAEBQGCcAHYc-MEEEGRRHcScAPG--YRLDDHQLcVS		
Factor IX-Bovine (res. 45 - 126)	YVDGDQcE-----SNPcLNG-GMcKTDINSYEcWcQAGF--EGTNcELD---ATcSIKNGRcKQFcKRDcDNKVcScTDG--YRLAEDQKScEP		
Factor X-Bovine (res. 44 - 126)	YKDGQcE-----GHPCcLNG-GHcKNGIGDYcTcAEGF--EGKNcEFSTR-EIcSLDWGGcDQFc-REERSEVRcScAHG--YVLGDDSKScVS		
		SILSVTAQQTGNcQPG--PLERSERSQTcACPAPFLVPSQGSIS	<i>a</i>
	<i>g</i> YEDDc-----GPGGcGSH-ARcYSDGETAECcLKGfARDGNLcS	RGRFc--RFGLc----ERDPKSHSSAcAEG--YTLSDRDKYcE	<i>b</i>
	<i>h</i> DIDEc-----VLARSDcPSTSSRcINTEGGYVcRcSEGfEGDGIScF	DVNEcATQNHGcTLGc-ENTPGSYHcTcPTG--FVLLPDGKQcH	<i>c</i>
Epidermal Growth Factor Homologues	<i>i</i> DIDEc-----QRGAHNCcAEN-AAcTWTEGGYNcTcAGRPSPPGRScPD	ELVScPGHVSkcSHGc-VLTSDGFRcIcPAG--SVLGRDGKTc	<i>d</i>
	<i>j</i> NSYPGcP-----SSYDGYcLNG-GVcMNIESLDSYcNcVIGYSGDRcQT	TGcSSPDNGGcSQIcLPLRPGSWcEcDcFPG--YDLQSDRKSAA	<i>e</i>
		GADPcLYRNGGcEHIC-QESLGTArcLREG--FVKAWDGKMLP	<i>f</i>

Fig. 2 Alignment of segments A and B from blood coagulation zymogens and 10 homologous segments from epidermal growth factor precursor (EGFP). Cysteine residues are shown in lower case. Underlined segments are predicted as turns by two different computer programs (broken lines predicted by only one of the two predictive programs). See Fig. 1 for locations of EGFP segments.

two separate exchanges with the factor X gene. This interpretation is in better agreement with the observation that all the homologous EGFP peptide segments nearer the amino terminus resemble unit B in factor X more than they do unit A, whereas the inverse is true of the segments nearer the carboxy terminus. If a DNA segment encoding an 80-residue previously duplicated twin had been the initiating material, then one might have expected, in whole or part, an alternating arrangement of homologous segments according to how much they resembled factor X_A and X_B, respectively.

The EGFP sequence was subjected to analysis by computer programs thought to be predictive of secondary structure^{10,11}. We were particularly interested in predicting 'turns', which are among the more accurately assignable features in proteins, for if the disulphides in each of the homologues exist in the same arrangements as occur in EGF¹², then they would be constrained to turn back on themselves three times. In fact, the remarkable agreement of predicted turns in the 10 homologues (Fig. 2) suggests that all of the units are tightly folded and that the disulphide pairings within them are similar. The six-cysteine 40-residue segments being examined here should not be confused with the six-cysteine 80-residue configurations commonly referred to as 'kringles', of which prothrombin contains two,

but factor IX, factor X or protein C apparently contain none¹³.

The 1,168-residue sequence of EGFP was also analysed by a computer program that provides a moving average of its hydropathy¹⁴. The profile closely resembled those of known membrane-attached glycoproteins¹⁵ in that a likely membrane-spanning segment was predicted for residues 1,039-1,058. In addition, a characteristic signal peptide was projected for residues 9-29, as suggested by Scott *et al.*². EGFP may exist on the surface of the cells of its origin, anchored by the spanning segment, and with residues 1,060-1,168 remaining inside the cell. Release of active EGF would occur as the result of limited proteolysis of the tethered precursor.

The known active EGF molecule corresponds to the 53-residue segment numbered 977-1,029. The segment is obviously released from the precursor (or some fragment thereof) by cleavage of an arginyl-asparagine bond at its amino terminus and an arginyl-histidine bond at its carboxy terminus. Neither of these dipeptidyl junctions occurs at equivalent positions around the other nine homologous segments, nor, do arginyl junctions of any type. In fact, if the disulphide pairing is analogous to that known to exist in EGF (segment *j*), then there is no possibility that tryptic-like cleavages would release EGF-like fragments from the nine homologues.

The topology of a computer-derived tree indicates that EGFP evolved before or about the time of the divergence of factors IX, X and protein C but definitely after their divergence from prothrombin. These particular blood coagulation zymogens belong to a small subset of vertebrate plasma proteins that contain clusters of the unusual amino acid γ -carboxyglutamic acid near their amino termini. The duplicated segments under discussion here are actually adjacent to the γ -carboxyglutamic acid region. Protein C and factors IX and X also contain the unusual amino acid β -hydroxyaspartic acid^{16,17}, and in this case the unusual amino acid is actually situated in segment A. None of the EGFP homologous unit has aspartic acid at the equivalent positions, however.

The divergence times can also be estimated from a consideration of the sequence differences between mouse and human EGF⁴. Knowing that rodents and primates last shared a common ancestor 80 Myr ago, and that the sequences of their EGFs differ in 17 of 53 residues, we can estimate an approximate rate of change. If we assume that the EGF-like units in the rest of the precursor protein have been changing at about the same rate, then the two most similar of these units, *c* and *d*, which differ by about 60%, diverged about 200 Myr ago. Two quite dissimilar units, *b* and *g*, are 80% different and by this measure would have diverged about 500 Myr ago. However, the (presumably) non-functional homologues may have been changing significantly faster than EGF itself. Nonetheless several of the homologous units resemble the zymogen segments more closely than does EGF. Given common ancestry, they have either been more rigorously conserved or have diverged on an independent and more recent occasion.

By either of these criteria EGF is an early vertebrate creation. The data agree with a gene encoding a segment of some primitive factor X-like molecule having been partially duplicated and translocated elsewhere in the genome. The transferred unit, whether corresponding to a 40- or an 80-residue segment, may have included parts of surrounding introns that subsequently became expressible as a result of the translocation. It seems reasonable that this new unit encoded a membrane-anchored protein from its inception and that limited proteolysis released a primitive EGF-like molecule. By chance the gene produced must have bound, however weakly, to an existing receptor for some other (growth) factor. Interestingly, factor IX binds to several cell types including fibroblasts¹⁸. From that point on the receptors and growth factors diverged in parallel, as is observed for many other biological systems. The existence of two similar sequences in the originally transposed unit would have facilitated further mismatching and tandem elongation.

It has recently been reported that a tumour growth factor from several sources is homologous with EGF¹⁹. The degree of similarity found is less than that observed between several of the EGF homologues and the corresponding segments in the zymogen clotting factors. Although the full sequences of these tumour growth factors are not known, the data available suggest that they are about 30% similar to EGF itself; the resemblance is less when the tumour growth factors are compared with any of the EGF homologues (*a-i*), suggesting that the tumour growth factors have not arisen directly from any of the other homologous units in EGF. Rather, it is consistent with the tumour growth factor and EGF arising as the result of duplication of a common ancestral gene. As such it can be expected that they will also have precursors that are anchored in the membranes of their cells of manufacture and will be activated by a scheme of extracellular proteolysis.

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Erythrocyte receptors for *Mycoplasma pneumoniae* are sialylated oligosaccharides of Ii antigen type

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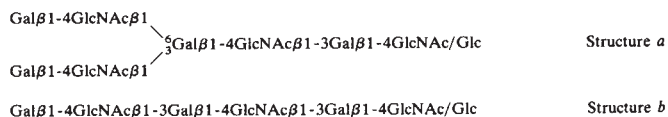
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Among the pathological effects in man following infection with *Mycoplasma pneumoniae* is a transient autoimmune disorder characterized by the presence of high-titre erythrocyte auto-antibodies (cold agglutinins)^{1,2}. These autoantibodies are usually directed against the carbohydrate antigen termed I (ref. 3) which consists of a branched oligosaccharide^{4,5}. The mechanism by which the anti-I antibodies are elicited is unknown. However, sialic acid-containing receptors have been implicated in the adherence of *M. pneumoniae* to erythrocytes and other cell types⁶, and both I and the related antigen i occur on erythrocytes in sialylated form⁷: i is the predominant antigen on fetal erythrocytes and I is predominant in adults⁸. Anti-I antibodies might arise in *M. pneumoniae* infection in response to a modification of the 'self' antigen-I as a result of its interaction with this agent^{9,10}. Here we report our study of the specificity of the interaction of *M. pneumoniae* with human erythrocytes. We found that this interaction is mediated by long chain oligosaccharides of sialic acid joined by α 2-3 linkage to the terminal galactose residues of poly-N-acetylglucosamine sequences of Ii antigen type.

Antigen I is built of repeating N-acetylglucosamine (Gal β 1-4GlcNAc) units as in structure *a* below^{4,5} and antigen i of linear poly-N-acetylglucosamine sequences¹¹ as in structure *b* (Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid; NeuGc, N-glycolylneuraminic acid).



The importance of sialic acid as a component of the erythrocyte receptor for *M. pneumoniae* was confirmed by the reduction of the binding of neuraminidase-treated erythrocytes to 10% of that of untreated erythrocytes (Table 1). The preferen-

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Table 1 Comparison of the binding of native, 'asialo' and resialylated erythrocytes to sheet cultures of *M. pneumoniae*

Erythrocytes	N-acetylneuraminic acid (nmol ml ⁻¹)		c.p.m. added ($\times 10^{-3}$)	c.p.m. bound ($\times 10^{-3}$)	% Binding
	Content	Incorporated			
Native	720		5,100	97.5	2
'Asialo'	40		4,500	8.9	0.2
Resialylated					
Gal β 1-4/3GlcNAc α 2,3 NeuAc		75	1,100	151	13.6
Gal β 1-4GlcNAc α 2,6 NeuAc		75	4,500	71	1.6
Gal β 1-3GalNAc α 2,3 NeuAc		260	3,900	19	0.5

Human blood group O erythrocytes of I type were desialylated^{38,39} using neuraminidase from *Vibrio cholerae* (Behringwerke) which released approximately 95% of the total sialic acid. Resialylation was achieved using donor CMP-NeuAc and one of the following purified enzymes: Gal β 1-3/4GlcNAc α 2-3-sialyltransferase^{40,41}, Gal β 1-4GlcNAc α 2-6-sialyltransferase^{40,41} and Gal β 1-3GalNAc α 2-3-sialyltransferase⁴². The N-acetylneuraminic acid (NeuAc) content (per ml of packed cells) was estimated as described previously³⁸. For radiolabelling, erythrocytes (1×10^9) were washed twice in Tris-buffered saline (TBS) containing 0.14 M NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.01 M Tris-HCl pH 7.2 and incubated at 37 °C for 45 min with 100 μ l of TBS containing 500 μ Ci sodium ⁵¹Cr-labelled chromate (350–600 mCi mg⁻¹ Cr) (Amersham). The cells were washed three times and resuspended in 100 μ l of TBS. ⁵¹Cr incorporation was in the range $1-10 \times 10^7$ c.p.m. per 1×10^9 erythrocytes as determined using a LKB Wallac 80,000 γ -counter. Sheet cultures were obtained using a strain of *M. pneumoniae*, MY 9862, which had been passed 10 times in mycoplasma liquid medium⁴³, once in hamster lung and then twice more in mycoplasma medium. Sheet cultures were prepared in 16 $\times$ 24 multiwell plates (Costar) by inoculating with 0.25 ml actively growing *M. pneumoniae* liquid culture (2.5×10^6 colour-changing units) per well containing 1 ml mycoplasma liquid medium. Plates were incubated at 37 °C until confluent sheets were obtained (3–4 days). The protein content of washed mycoplasma sheets, after solubilization in 1 M NaOH, was 20–30 μ g per well; this was determined⁴⁴ using bovine serum albumin as a standard. For binding assays, the mycoplasma monolayers or uninoculated wells containing medium alone were washed twice with 1 ml TBS, then ⁵¹Cr-labelled erythrocytes (1×10^7 cells in 300 μ l) were added (in triplicate). The c.p.m. added varied with the erythrocyte preparations due to their differing incorporation of ⁵¹Cr. The plates were incubated at 37 °C for 1 h and unattached erythrocytes were removed by washing each well with 3 $\times$ 1 ml of TBS. The attached erythrocytes in each well were solubilized in 1 ml of 1 M NaOH and the radioactivity counted for 30 s. The radioactivity bound to medium control wells (0.2–0.5% of c.p.m. added) was subtracted from that bound to the mycoplasma monolayers.

tial binding of *M. pneumoniae* to sialic acid joined by α 2–3 linkage to galactose was clearly shown with the resialylated erythrocytes as follows. First, compared with 'asialo' erythrocytes, those having the NeuAc α 2–3Gal β 1–4/3GlcNAc sequence had a 68-fold greater binding while those having the NeuAc α 2–6Gal β 1–4GlcNAc and NeuAc α 2–3Gal β 1–3GalNAc sequences had only 8- and 2.5-fold greater binding, respectively (Table 1). Thus, erythrocytes having the NeuAc α 2–3Gal β 1–4/3GlcNAc sequence had 8.5-fold greater binding than those with the NeuAc α 2–6Gal β 1–4GlcNAc sequence. Second, compared with the native erythrocytes, the NeuAc α 2–3Gal β 1–4/3GlcNAc-derivatized erythrocytes showed almost sevenfold greater binding despite their lower sialic acid content. A major sialylated product obtained with the Gal β 1–4/3GlcNAc α 2–3-sialyltransferase (unpublished observations) as with the Gal β 1–4GlcNAc α 2–6-sialyltransferase¹², corresponds to band 3 glycoprotein. Thus, the supranormal binding of erythrocytes derivatized using the Gal β 1–4/3GlcNAc α 2–3-sialyltransferase can be explained in terms of the derivatization of band 3 oligosaccharides that are not naturally sialylated to this extent. Third, erythrocytes derivatized using the Gal β 1–3GalNAc α 2–3-sialyltransferase showed negligible binding above the 'asialo' erythrocytes despite their greater than six-fold content of sialic acid. This enzyme acts predominantly on the 0-glycosidically linked oligosaccharides of glycophorin A¹².

The specificity of the *M. pneumoniae*–erythrocyte interaction was next investigated using oligosaccharides and glycoproteins as inhibitors of binding (Table 2). The preference for sialic acid α 2–3-linked rather than α 2–6-linked to galactose as a receptor, sequence was confirmed with the two α 2–3-linked isomers (Table 2, structures *d* and *f*) which were seven and nine times, respectively, more active as inhibitors than the corresponding α 2–6 isomers (Table 2, structures *e* and *g*). Moreover, sialic acid α 2–6-linked to N-acetylgalactosamine was ruled out as a receptor sequence for *M. pneumoniae* as none of the three submaxillary mucins tested gave inhibition. These glycoproteins

are known to contain sialic acid predominantly α 2–6-linked to N-acetylgalactosamine^{13–16}.

In its strong preference for sialic acid α 2–3-linked to galactose residues, *M. pneumoniae* differs from *Mycoplasma gallisepticum* which does not distinguish between sialic acid α 2–3-linked to galactose or α 2–6-linked to N-acetylgalactosamine¹⁷. In contrast to our findings with *M. pneumoniae* (Table 2), the submaxillary mucins of ovine, bovine and porcine origins were found¹⁷ to be effective inhibitors of the binding of *M. gallisepticum* to erythrocytes.

The trisaccharide sequence α 2–3-N-acetylneuraminosyllactosamine (Table 2 as in structure *d*) was almost three times as active as α 2–3-N-acetylneuraminosyllactose (structure *f*) indicating that N-acetylglucosamine is part of the receptor sequence. The inhibition experiments summarized in Table 2 show the strikingly strong inhibitory activity of oligosaccharides and glycoproteins containing the repeating N-acetylglucosamine sequence. Thus, oligosaccharide structure *c* containing two N-acetylglucosamine units was five times more active than structure *d* with a single unit. Similarly, among the glycoproteins tested, the most potent inhibitors were the polyglycosyl peptides of human erythrocytes (largely derived from bands 3 and 4.5) and the bovine erythrocyte glycoprotein GP-2, both of which are rich in branched poly-N-acetylglucosamine sequences of I antigen type (refs 18–20 and Y. Suzuki, H. C. Gooi & T.F., unpublished observations). Human glycophorin A and α 1-acid glycoprotein were substantially less active and fetuin was inactive, in accordance with their lack of poly-N-acetylglucosamine sequences^{21–26}. Inhibition experiments with glycolipids (to be described elsewhere) have also indicated that sialylated poly-N-acetylglucosamine sequences are the preferred sequences for *M. pneumoniae* binding and that the sialic acid residues can be of N-acetyl or N-glycolyl type.

Although our results and those of others^{27,28} have shown that solubilized glycophorin A as well as glycolipids and oligosaccharides having short chain sequences can inhibit the binding

of erythrocytes to *M. pneumoniae*, the following experiments showed that they do not serve as receptors for this agent on intact erythrocyte membranes. First, glycophorin-deficient cells, termed En(a⁻), showed normal mycoplasma binding (Table 3); these cells have a 30–50% reduction in their sialic acid levels^{29,30}. Second, following Pronase digestion of erythrocytes, which results in the loss of almost all the glycosylated portions of the major sialoglycoproteins (D. J. Anstee & M. J. A. Tanner, personal communication) but not of band 3 protein³¹, the binding

Table 2 Inhibition of binding of ⁵¹Cr-labelled erythrocytes to sheet cultures of *M. pneumoniae* by oligosaccharides and glycoproteins

Designation	Inhibitors	Concentration*
c	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-Asn α2,3 NeuAc	16
d	Galβ1-4GlcNAc-Asn α2,3 NeuAc	88
e	Galβ1-4GlcNAc-Asn α2,6 NeuAc	640
f	Galβ1-4Glc α2,3 NeuAc	245
g	Galβ1-4Glc α2,6 NeuAc	2,136
h	NeuAc	NA
j	Galβ1-4Glc [†]	NA
	Glycoproteins	
	Polyglycosyl peptides	0.9
	Bovine erythrocyte sialoglycoprotein (GP-2)	2.5
	Glycophorin A	12.6
	α ₁ -acid glycoprotein	47.7
	Bovine fetuin	NA
	Submaxillary mucins; bovine, ovine, porcine	NA
	Bovine serum albumin†	NA

Structures c, d and e (asparaginyl oligosaccharides) were isolated from the urine of patients with aspartylglucosaminuria (J.-C.M., J. Montreuil, G. Strecker, H. van Halbeek and J. Vliegthart, in preparation). Structure g, NeuAcα2-6 isomer of sialosylactose, isolated from human milk, was given by the late Dr Adeline Gauhé. Structure f, NeuAcα2-3 isomer of sialosylactose, and structure j, N-acetylneuraminic acid and lactose, were from Sigma. The preparation of α2-6 isomer of sialosylactose was found to contain 11% free N-acetylneuraminic acid and the preparation of α2-3 isomer contained 10% α2-6 isomer as assessed by HPLC⁴⁵. The inhibition values shown for these two isomers of sialosylactose have been corrected to allow for the impurities. Polyglycosyl peptides, obtained by pronase digestion of human erythrocyte membranes¹⁸, were given by Dr J. Finne; the sugar composition and their molar ratios were fucose 3.6: mannose 3.0: galactose 13.9: N-acetylglucosamine 15.5: N-acetylneuraminic acid 2.8, (approximately 3 mol mannose per mol glycopeptide). The bovine erythrocyte sialoglycoprotein, GP-2, which is a strong inhibitor of haemagglutination by Sendai virus⁴⁶, was given by Dr Y. Suzuki. This glycoprotein contains 70% carbohydrate by weight; the monosaccharides N-acetylglucosamine, N-acetylglucosamine, galactose and sialic acid (N-glycolylneuraminic acid 96%; N-acetylneuraminic acid 4%) are present in molar ratios of 1.0:4.0:5.2:2.9. The backbone sequence is deduced to be a branched poly-N-acetylglucosamine structure from the strong I antigen activity expressed on asialo GP-2 (Y. Suzuki, H. C. Gooi & T.F., unpublished observations). Glycophorin A from human erythrocyte membranes⁴⁷ was a gift of Dr C. G. Gahmberg. Human α₁-acid glycoprotein was a gift from Dr K. Schmid. Bovine fetuin type IV was from Sigma. Ovine submaxillary mucin was from Dr D. van den Eijnden; bovine and porcine submaxillary mucins from Drs A. Corfield and R. Schauer; and bovine serum albumin (fraction V) from Miles Laboratories. Inhibition of binding assays were performed as follows: washed mycoplasma monolayers were preincubated with 300 μl of serial dilutions of inhibitors (in triplicate) in TBS at 37 °C for 1 h before the addition of 300 μl of ⁵¹Cr-labelled erythrocytes (1 × 10⁶ cells containing 1–10 × 10⁴ c.p.m.). All subsequent steps were as for the binding assays (Table 1). Inhibition was calculated as the per cent reduction in binding relative to control wells which contained no inhibitor.

NA, No inhibition at the highest levels tested as follows: NeuAc 3,236 nmol; bovine fetuin 292 nmol sialic acid; submaxillary mucins; bovine 1,070, ovine 1,050 and porcine 470 nmol sialic acid. The relatively high inhibitory activities of the glycoproteins reflect the cooperative effects of multivalence of their carbohydrate chains.

* Concentration expressed as nmol sialic acid ml⁻¹ giving 50% inhibition.

† Lactose, structure j (2,900 nmol oligosaccharide) and the non-glycosylated protein; bovine serum albumin (1 mg) served as non-sialylated controls.

of mycoplasma was not reduced and was even slightly increased (Table 3). The carbohydrate chains of band 3 protein, but not those of the sialoglycoproteins, are known to contain poly-N-acetylglucosamine sequences having I antigen activity^{19,20}. Third, erythrocytes treated with endo-β-galactosidase showed reduced binding to the *M. pneumoniae* sheets (Table 3), indicating that long chain, poly-N-acetylglucosamine sequences of Ii type³² are the main receptors for this agent. The reduction in binding reflected the content of endo-β-galactosidase-susceptible poly-N-acetylglucosamine sequences in the three cell types as follows: the binding of normal and En(a⁻) erythrocytes of I type was reduced to 44% and 43%, respectively, of untreated cells (Table 3), in accordance with the predominance in these cells of branched oligosaccharides of poly-N-acetylglucosamine type which are only partially susceptible^{20,33} to endo-β-galactosidase. The availability of the variant erythrocytes of i-antigen type provided the strongest evidence for the importance of sialylated poly-N-acetylglucosamine sequences as receptors for *M. pneumoniae*. Cells of i type have a high content of linear poly-N-acetylglucosamine sequences which are more susceptible to digestion with endo-β-galactosidase than the corresponding branched sequences on erythrocytes of I type^{20,33}. The binding of these cells was reduced by 85% following treatment with endo-β-galactosidase. This is remarkable as only 5% of the total erythrocyte sialic acid is released by this glycosidase²⁰. Thus, minor sialylated oligosaccharides of the poly-N-acetylglucosamine series, such as those carried by glycoprotein bands 3

Table 3 Effect of Pronase and endo-β-galactosidase treatment on binding of ⁵¹Cr-labelled erythrocytes of OI, Oi and En(a⁻) types to sheet cultures of *M. pneumoniae*

Erythrocytes	c.p.m. added (×10 ⁻²)	c.p.m. bound (×10 ⁻²)	% Binding
OI-untreated	800	57	7.1
Pronase	800	72	9.0
Endo-β-galactosidase	1,000	31	3.1
Oi adult-untreated	1,000	68	6.8
Endo-β-galactosidase	600	6	1.0
En(a ⁻)-untreated	1,000	71	7.1
Endo-β-galactosidase	1,000	30	3.0

Blood group OI erythrocytes were from a normal donor. Blood group O erythrocytes of the rare i phenotype⁴⁸ were from an adult deficient in I antigen [Oi (adult)]. Erythrocytes from an adult genetically deficient in the major erythrocyte sialoglycoprotein, glycophorin A (designated En(a⁻)⁴⁹) were given by Dr D. Anstee. For treatment with endo-β-galactosidase, 150 μl packed erythrocytes were washed three times in PBS and incubated at 37 °C for 3 h with 50 μl of PBS containing 6 mU of endo-β-galactosidase from *Bacillus fragilis*⁵⁰. This enzyme cleaves poly-N-acetylglucosamine-type oligosaccharides containing the sequence GlcNAcβ1-3Galβ1-. The cells were then washed three times in PBS. For treatment with Pronase (Sigma, type VI), 150-μl packed cells were incubated at 37 °C for 30 min with 150 μl PBS containing 2 mg ml⁻¹ Pronase; they were then washed three times in PBS. Radiolabelling of the untreated and enzyme-treated erythrocytes with ⁵¹Cr and binding to mycoplasma monolayers was as described in the legend to Table 1; 3 × 10⁶ erythrocytes were added to each monolayer.

and 4.5, and glycolipids²⁰ are the main receptors for *M. pneumoniae* rather than the carbohydrate chains of the major sialoglycoproteins which are not susceptible to digestion by endo-β-galactosidase.

The present studies further support the concept that the post-infective autoantibodies (anti-I) elicited following infection with *M. pneumoniae* occur as a result of the interaction of I antigen with the mycoplasma and the presentation of this 'self' antigen in an immunogenic form to the immune system of the host. This was first suggested in 1969 when we (T.F., D.T.R. and colleagues) immunized rabbits with human group OI erythrocytes which had been treated with live *M. pneumoniae* and about 30% of the rabbits developed high titres of anti-I antibodies³⁴. More recently, we have obtained evidence that *M. pneumoniae* cultures absorb I-active glycolipids from serum-containing culture media. These observations now account for previous reports of I-antigen activity associated with laboratory strains of *M. pneumoniae*^{35,36}.

In view of the observation that *M. pneumoniae* can react with erythrocytes of both I and i type, it is intriguing that anti-I

antibodies rather than anti-i antibodies are elicited following infection with this agent. A possible explanation is that *M. pneumoniae* infection occurs usually in children and adults rather than neonates³⁷ and the i type oligosaccharides of fetal erythrocytes are largely replaced by I type structures in the first year of life^{8,20}. Thus *M. pneumoniae* would encounter and adhere to I- rather than i-type erythrocytes and possibly other cells of I type in the inflamed bronchial epithelium.

The principles established with the *M. pneumoniae*-erythrocyte receptor system could apply to a variety of receptor systems where inhibition and cell binding data have been difficult to reconcile. Also, the complexing of adhesive molecules of infective agents to specific saccharides of host cell membranes may be an important mechanism for the eliciting of auto-antibodies with anti-receptor specificities.

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Stability of expression-linked surface antigen gene in *Trypanosoma equiperdum*

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African trypanosomes evade clearance in immune-competent hosts by periodically replacing their major surface glycoprotein with an antigenically different glycoprotein¹⁻⁴. Expression of many of these variant surface glycoproteins (VSGs) is associated with the duplication and transposition of silent basic copy genes (BCs) into unlinked genomic expression sites⁵⁻¹⁰. The new expression-linked VSG gene copies (ELCs) are oriented with their 3' ends proximal to chromosome telomeres¹¹⁻¹⁴. Other VSG genes are activated without the production of an ELC^{10,15-18}. The 3' ends of these VSG genes are near chromosome telomeres both when they are active and when they are inactive. Recently, we have shown that activation of the VSG-1 gene in the BoTaR (Bordeaux trypanozoon antigen repertoire) serodeme of *Trypanosoma equiperdum* involves the duplication and transposition of a telomeric BC gene into one of at least three unlinked telomeric sites⁹. Here we show that the VSG-1 ELC is inactivated but not eliminated in some antigenic variants derived from a VSG-1 expressor. In addition, a subsequent variant that again expresses VSG-1 has not reactivated the residual VSG-1 ELC (R-ELC), but instead contains a new, active VSG-1 ELC in an unlinked telomeric site. These results show that the simple presence of an ELC in a potential expression site is not sufficient for its expression.

We examined the fate of the VSG-1 ELC in *T. equiperdum* variants isolated from a rabbit infected with VSG-1 expressor BoTat-1 (Bordeaux trypanozoon antigen type-1). Figure 1a explains the genealogy of these trypanosome clones. Trypanosomes expressing VSG-1 always appear in the first peak of parasitaemia in rabbits infected with any variant from the BoTaR serodeme of *T. equiperdum*⁴. VSG-2 and -4 expressors also appear very early, whereas VSG-20, -28 and -78 expressors appear later in such infections. Figure 1b compares the hybridization profiles of the DNAs from these trypanosomes after digestion with *Bam*HI endonuclease, which does not cleave within the VSG-1-coding sequence, and hybridization to a ³²P-labelled VSG-1-specific cDNA clone.

As described previously⁹, the profile of BoTat-1 showed four fragments, one of which contained the VSG-1 ELC (14 kilobases, kb), whereas the profiles of BoTats-20, -28 and -78 each showed only three fragments hybridizing to the VSG-1 cDNA. Surprisingly, the hybridization patterns of BoTats-2 and -4 also contained a fourth VSG-1-related band. DNA from BoTat-4⁽²⁰⁾, a second VSG-4-expressing variant derived from BoTat-20, showed only three *Bam*HI fragments hybridizing to the VSG-1 probe (Fig. 1b). Thus, the supplementary VSG-1-related sequences in the DNAs of BoTats-2 and -4 were not necessary for expression of the early VSGs.

The above results suggested that the supplementary VSG-1 sequences in BoTats-2 and -4 represented inactive residual VSG-1 ELCs (R-ELCs). To examine this possibility, restriction maps of the regions containing the apparent R-ELCs in these variants were constructed as described in Fig. 2 legend. The 5' genomic environments of the R-ELCs seemed to be identical to that of the original VSG-1 ELC in BoTat-1, at least as far as the *Sal*I site more than 9 kb upstream from the gene. The restriction maps of the coding regions of the R-ELCs and the BoTat-1 ELC were also identical. Moreover, like both the

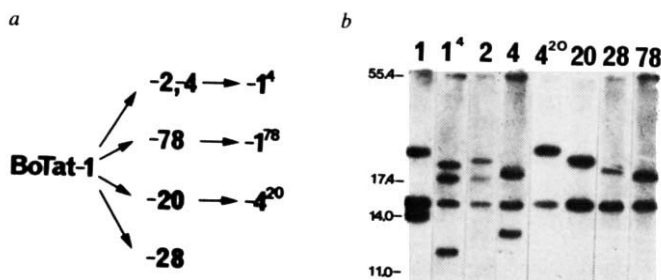


Fig. 1 *a*, Genealogy of trypanosome variants. All the *T. equiperdum* variants described here were derived from BoTat-1 as described by Capbern *et al.*⁴. Arrows represent infection of a rabbit with the designated variant. The variants were then isolated from the rabbits and cloned in mice. The variants are labelled here and in subsequent figures according to their numbers: 2 refers to BoTat-2 which expresses VSG-2, etc. BoTats-2 and -4 were isolated from the same rabbit. BoTat-1⁽⁴⁾ was isolated from a rabbit infected with BoTat-4. The rabbit infected with BoTat-20 from which BoTat-4⁽²⁰⁾ was isolated, was immunized against VSG-1 to prevent passage through a VSG-1-expressing intermediate. *b*, *Bam*HI restriction profiles of VSG-1-related sequences in the DNAs of various variants. DNA was isolated from each variant, digested with *Bam*HI which does not cut the VSG-1-coding sequence, electrophoresed in a 0.5% agarose gel, blotted to nitrocellulose filters and hybridized to ³²P-labelled VSG-1-specific cDNA plasmid pTe 1.6 as described previously⁹. The numbers above the lanes denote the variants from which the DNA was taken. The large (>60 kilobase pair) fragment is present in all of these variants, but is partially degraded in some of the DNA preparations. Molecular weight standards were from a *Bgl*II digest of coliphage T4 DNA and sizes are given in kb.

VSG-1 ELC and BC in BoTat-1¹⁴, the 3' ends of the VSG-1 R-ELCs were proximal to apparent chromosome telomeres. This was inferred from the presence of 'universal' restriction sites at the presumed chromosome termini in the DNA sequences flanking the 3' ends of these R-ELCs (see maps in Fig. 3) and from the selective sensitivity of these 3'-specific fragments to *Bal*31 exonuclease (data not shown). The only differences observed in the maps of the original VSG-1 ELC and the R-ELCs were in the lengths of the DNA sequences between the VSG-1-coding sequence and the apparent chromosome telomeres.

To assess the level of activity of the R-ELCs in BoTats-2 and -4, the trypanosomes were screened by immunofluorescence with anti-VSG-1 antibody and their purified RNAs were examined by Northern filter hybridization with VSG-1-specific cDNA probes; no VSG-1 glycoprotein or VSG-1 mRNA was detected (data not shown). Finally, because it was shown recently that chromatin containing the ELC of an expressed VSG gene is selectively sensitive to DNase I⁷, we examined the DNase I sensitivity of chromatin containing the VSG-1 R-ELCs in BoTats-2 and -4. Chromatin was isolated from BoTats-1, -2 and -4, treated with DNase I, and analysed as described in Fig. 3 legend. As expected from previous work⁹, the 8.6-kb *Sac*I fragment containing the 5' end of the VSG-1 ELC in BoTat-1 was selectively degraded by the DNase I treatment (Fig. 3*a*). In contrast, the 8.6-kb fragments containing the 5' end of the VSG-1 R-ELC in BoTat-2 (not shown) and BoTat-4 (Fig. 3*b*) were not selectively sensitive to DNase I. From these results we conclude that the VSG-1 R-ELCs are inactive and that their inactivation was accompanied by alterations of the chromatin containing the gene.

As the VSG-1 ELC was inactivated but not lost on antigenic variation to BoTats-2 and -4, we asked whether it would be reactivated after a change back to VSG-1 expression. Thus, a rabbit was infected with BoTat-4 and the VSG-1-expressing variant BoTat-1⁽⁴⁾ was isolated. The hybridization profile of the VSG-1-specific sequences in BoTat-1⁽⁴⁾ DNA after cleavage with *Bam*HI is shown in Fig. 1*b*. Clearly, BoTat-1⁽⁴⁾ contained two VSG-1-specific sequences not present in BoTats-20, -28

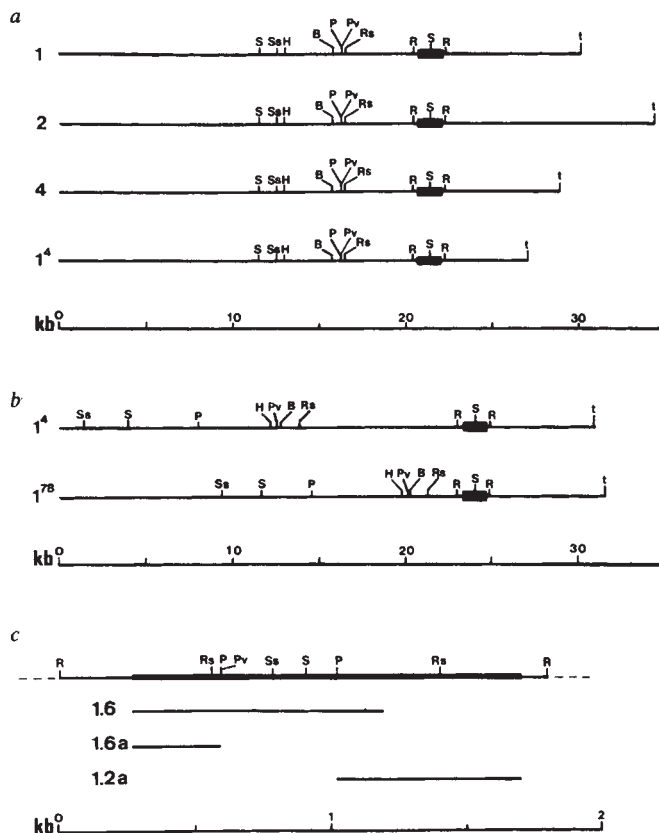


Fig. 2 Restriction maps of DNA containing VSG-1 genes. *a*, Maps of VSG-1 ELC in BoTat-1 and VSG-1 R-ELC in BoTats-2, -4 and -1⁽⁴⁾. *b*, Maps of active VSG-1 ELCs in BoTats-1⁽⁴⁾ and -1⁽⁷⁸⁾. *c*, Map of expanded coding sequence of the VSG-1 gene. The derivations of the various probes have been described previously⁹ and are shown beneath the expanded map: 1.6, pTe 1.6; 1.6a, 5'-specific pTe 1.6a; 1.2a, 3'-specific pTe 1.2a. The maps were generated by treating the DNAs with single or multiple restriction endonucleases and hybridizing separately with ³²P-labelled 3'- or 5'-specific VSG-1 probes. Sites mapping within the sequences homologous to the VSG-1 cDNA clones (solid boxes) are not shown in *a* or *b* but are given in the expanded map in *c*. Transcription is from left to right. Sites that are 'universally' sensitive to restriction endonucleases and are presumed chromosome telomeres are marked (t). Symbols: S, *Sa*I; Ss, *Sst*I or isoschizomer *Sac*I; H, *Hind*III; B, *Bam*HI; P, *Pst*I; Pv, *Pvu*II; Rs, *Rsa*I; R, *Eco*RI.

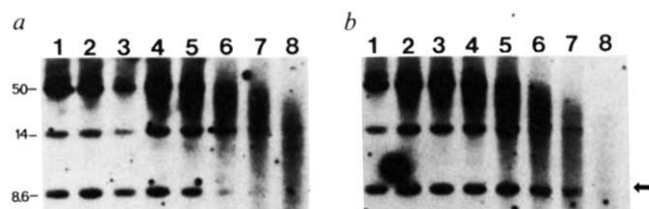
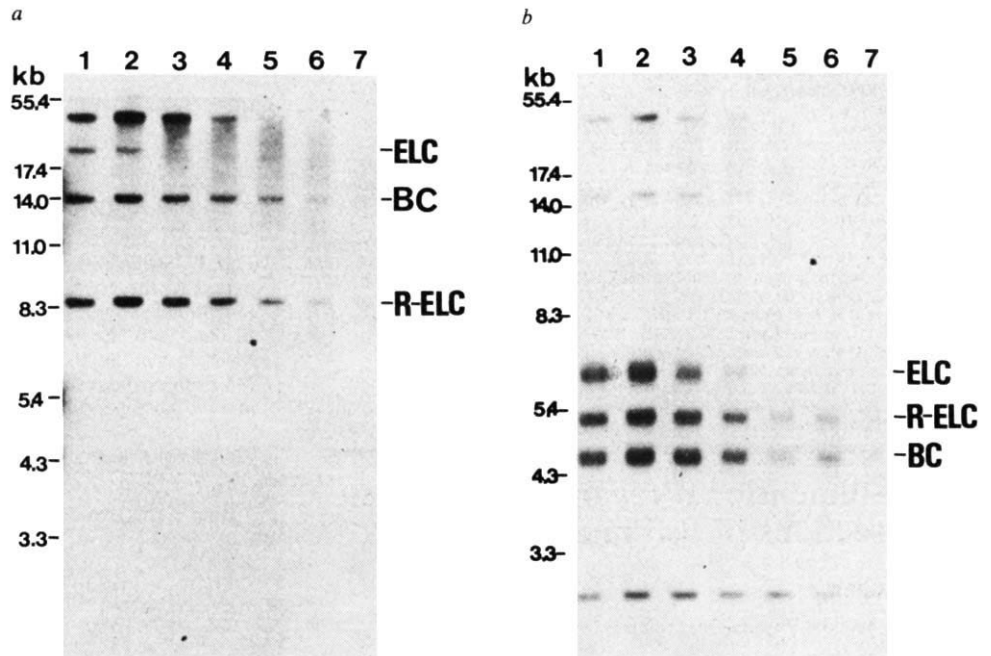


Fig. 3 DNase I-sensitivity of VSG-1 R-ELCs. *a*, DNA from BoTat-1; *b*, DNA from BoTat-4. The procedure for DNase I treatment of trypanosome chromatin has been described in detail elsewhere⁷. Briefly, nuclei were isolated from 2×10^9 trypanosomes and treated at 20 °C with 80 ng ml⁻¹ RNase-free DNase I (Worthington Biochemicals). Aliquots were taken at 0 min (lane 1), 0.5 min (lane 2), 1 min (lane 3), 2 min (lane 4), 5 min (lane 5), 10 min (lane 6), 20 min (lane 7) and 40 min (lane 8). The DNA was extracted, treated with *Sac*I, electrophoresed in a 0.6% agarose gel, blotted and hybridized with ³²P-labelled 5'-specific cDNA probe pTe 1.6a. The arrow shows the position of the 8.6-kb fragment containing the 5' end of the VSG-1 ELC in BoTat-1 or the VSG-1 R-ELC in BoTat-4. Sizes of the fragments are in kb.

Fig. 4 DNase I-sensitivity of new VSG-1 ELC and R-ELC in BoTat-1⁽⁴⁾. *a*, Sensitivity of 5'-specific sequences; *b*, sensitivity of 3'-specific sequences. Nuclei were isolated from BoTat-1⁽⁴⁾ and treated with DNase I as described in Fig. 3 legend. Aliquots were taken at 0 min (lane 1), 0.5 min (lane 2), 1 min (lane 3), 2 min (lane 4), 5 min (lane 5), 10 min (lane 6) and 20 min (lane 7). The DNA was extracted, cut with *Sac*I, electrophoresed and blotted as described in Fig. 3 legend. The blot in *a* was hybridized with the 5'-specific VSG-1 probe pTe 1.6a; that in *b* was hybridized with the 3'-specific VSG-1 probe pTe 1.2a. The positions of the fragments containing the 3' or 5' ends of the new VSG-1 ELC (ELC), the residual VSG-1 ELC (R-ELC) and the 3' end of the VSG-1 basic copy (BC) are shown. Molecular weight standards are from a *Bgl*III digest of coliphage T4 DNA.



or -78. Restriction analysis showed that the 5' genomic environment of one of these VSG-1 copies was identical to that of the BoTat-1 ELC and the VSG-1 R-ELCs in BoTats-2 and -4 (Fig. 2a). The DNA flanking the 3' end of this VSG-1 gene was associated with an apparent chromosome telomere. We conclude, therefore, that this VSG-1 gene represents the VSG-1 R-ELC and that it was not deleted during the processes that produced BoTat-1⁽⁴⁾.

The second supplementary VSG-1 sequence in BoTat-1⁽⁴⁾ was newly produced in this variant. The 5' flanking regions of this VSG-1 gene resembled the 5' flanking region of the previously described VSG-1 ELC in BoTat-1⁽⁷⁸⁾ (Fig. 2b). However, it contained a 7-8-kb restriction endonuclease-insensitive sequence that is not present in the corresponding region of the BoTat-1⁽⁷⁸⁾ ELC. This was not surprising in view of previous reports that the 5' environments of different ELCs in the same genomic site differ by the presence of variable length DNA sequences that are insensitive to restriction enzymes^{9,10,13,19-22}. The 3' end of the newly produced VSG-1 gene in BoTat-1⁽⁴⁾ was again proximal to a chromosome telomere and thus cannot be closely linked to the telomeric VSG-1 R-ELC in this variant.

To determine which of the VSG-1 gene copies in BoTat-1⁽⁴⁾ were active, chromatin was isolated and analysed with DNase I as described above. With a 5'-specific VSG-1 cDNA probe, only the 22-kb *Sac*I fragment containing the 5' end of the newly produced VSG-1 gene was preferentially DNase I-sensitive; the 8.6-kb fragment containing the 5' end of the VSG-1 R-ELC was stable (Fig. 4a). Similar experiments using 3'-specific VSG-1 cDNA plasmid as probe showed that the fragments containing the 3' ends of the VSG-1 BC and R-ELC were slightly sensitive to DNase I treatment, but that the fragment containing the 3' end of the new VSG-1 gene was very sensitive to such treatment (Fig. 4b). We have shown previously that the telomeric DNA associated with silent VSG genes is slightly sensitive to DNase I treatment²³. These results suggested that only the new VSG-1 copy in BoTat-1⁽⁴⁾ is transcribed. Thus, in BoTat-1⁽⁴⁾, the VSG-1 R-ELC was not reactivated and a new, active VSG-1 ELC was created.

Our observations show that the VSG-1 ELC in BoTat-1 was inactivated *in situ* during the antigenic variations which produced BoTats-2, -4 and -1⁽⁴⁾. This demonstrates that the simple presence of a VSG-1 ELC in a potential expression site is not sufficient for its expression. The new, active VSG-1 ELC and the inactive R-ELC in BoTat-1⁽⁴⁾ were shown to be linked to

different chromosome telomeres. Similarly, the ELCs of the VSG-2 and VSG-4 genes were not closely linked to the VSG-1 R-ELCs in BoTats-2 and -4 (unpublished data). These results reinforce our previous assertion that there are multiple telomeric sites which can accept VSG-1 ELCs.

Overall, our results with *T. equiperdum* argue that VSG genes are activated by duplication and transposition into one of several telomeric acceptor sites which are subject to secondary control mechanisms. Thus a VSG gene could be activated either by direct replacement of the previously active ELC or by transposition into an inactive acceptor site which can subsequently be activated. This model explains how some VSG genes are apparently activated by a simple duplicative transposition (direct replacement of an active ELC) and how other VSG genes are activated without obvious duplication (activation of an inactive acceptor site containing a previously duplicated VSG gene). Loss of an ELC, as observed for the VSG-1 ELC after variation from BoTat-1 to BoTats-20, -28 and -78, would occur by its replacement in the acceptor site with a newly duplicated VSG gene copy. Conclusions similar to these have been reached by Young *et al.*²⁴

The mechanisms which activate the duplicated VSG genes in the various acceptor sites are not yet understood. Translocation of a VSG gene in an acceptor site to a position *cis* to an 'expression element' via a reciprocal recombination 10-30 kb upstream from the VSG gene would explain our observations of multiple acceptor sites and the maintenance of the VSG-1 R-ELCs. Similarly, an 'expression element' could be recombined into a position 3' to the VSG gene-containing acceptor site. Other mechanisms such as DNA modification or a system of repressors and inducers are also possible factors controlling VSG genes in a finite number of telomeric acceptor sites.

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Three-dimensional reconstruction from tilted sections of fish muscle M-band

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Fish muscle provides a particularly suitable specimen for studying the organization of thick filaments in vertebrate striated muscle because the filaments are arranged with a single orientation on a well ordered hexagonal lattice¹. The M-band consists of sets of cross-links, which join the myosin filaments in the middle of the A-band. Previous work¹ concluded that the fish muscle M-band had the local symmetry of the dihedral point group 32. We present here a quantitative analysis of transverse sections of the M-band, using general methods² developed for crystalline layers, to combine various tilted views. The three-dimensional map computed to a resolution of about 70 Å confirms previous findings¹; it shows new features of the thick filament structure in the M-band region and provides new information on the use of three-dimensional reconstruction from sectioned biological material. The accompanying paper³ describes a novel technique of three-dimensional reconstruction of the fish M-band from a single view of a slightly oblique plastic section.

In fish muscle the thick filaments are arranged in rotational register¹, making the M-band crystalline. There are three main axial levels of M-bridges within this M-band⁴: M1 in the middle, with M4 and M4' about 220 Å on either side (Fig. 1). The bands at M6 and M6', about 220 Å outside M4 and M4', are very weak in the plaice fin muscle studied here. From a study¹ of very thin slightly oblique transverse sections, three distinct structures were identified, which were thought to be associated with the M1, M4 and M6 bands seen in longitudinal sections (Fig. 1). At M1 there were straight M-bridges while at M4 and M4' the thick filament backbone comprised three subunits with bean-shaped M-bridges. The structure showed a clear hand which reversed from M4 to M4' and the symmetry of the M-band corresponded to the dihedral point group 32.

The micrograph in Fig. 2 shows an example of an M-band cross-section used for three-dimensional reconstruction. The thick filament spacing was about 350 Å and this value was used throughout, although the value in the native muscle would be somewhat larger. The section, estimated as about 600 Å thick from the interference colour during microtomy, should include the whole of a centrally located M-band from M4 to M4' (440 Å). Micrographs were taken at tilt angles from -60° to +60° in steps of 5° about two mutually perpendicular axes. The

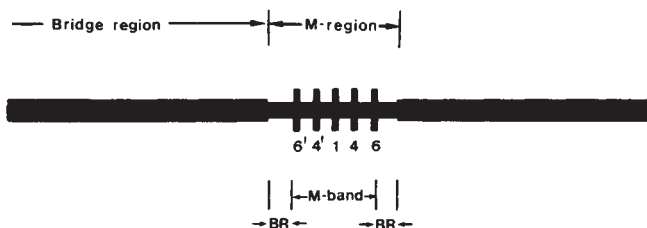


Fig. 1 Schematic drawing of part of a thick filament showing details of the myosin crossbridge-free M-region as it is seen in longitudinal sections of the A-band⁴. The M-region comprises the M-band and the bare region (BR). The three main levels of M-bridges in the M-band are M1 in the middle with the M4' and M4 bridges about 220 Å to either side. Additional density is seen in some muscles at M6 and M6', about 220 Å outside M4 and M4'.

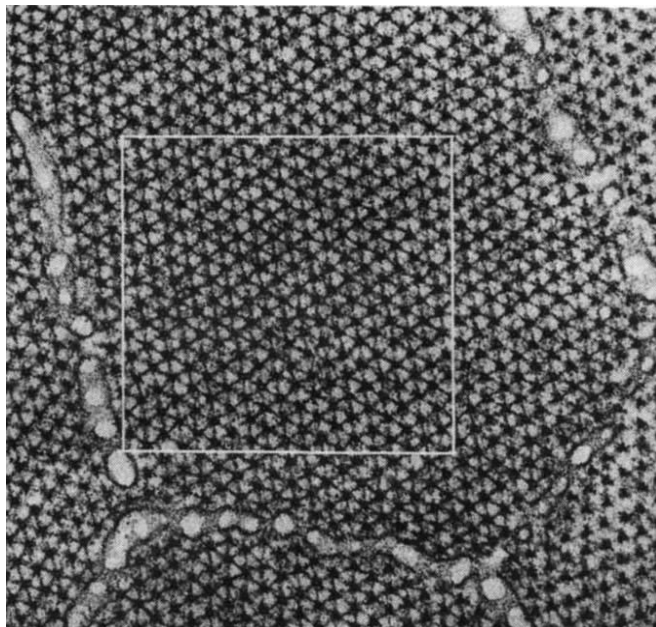


Fig. 2 Transverse section of the plaice fin muscle M-band ($\times 80,000$). The boxed area was used for reconstruction. The muscle was processed and embedded in Araldite as described previously¹. Transverse sections of silver-grey interference colour were cut with a diamond knife which, from past experience using section folds, corresponds to a thickness of about 600 Å. The sections were stained with uranyl acetate and Reynolds lead citrate. For electron microscopy, a JEOL 200CX fitted with a rotation goniometer was used. Micrographs at a magnification of $\times 20,000$, were obtained by tilting the section in steps of 5° from -60° to +60°, covering the low tilt angles first in order to stabilise the section. The section was then rotated by 90° and another tilt series obtained. The best areas were selected by optical diffraction.

total exposure was consequently large but it appeared that the section was relatively stable after the initial high dose exposure.

Fourier transforms were computed of each view and a three-dimensional set of data was constructed using standard programs², assuming minimal P1 symmetry in the specimen. Figure 3 shows data for some of the stronger lattice lines. The transform of the untilted view showed strong 3-fold rotational symmetry when the appropriate phase origin was found on the thick filament, although the lattice was distorted by compression of about 10% in the direction of sectioning. The three-dimensional reconstruction computed with no assumption about symmetry also showed a similarly striking 3-fold rotation axis, though again with compression of the lattice. After correcting for the compression, the data were therefore 3-fold averaged and a further reconstruction computed. It is this 3-fold averaged map which will be described.

The computed thickness of the section in the reconstruction was only about 300 Å, a value much lower than the initial

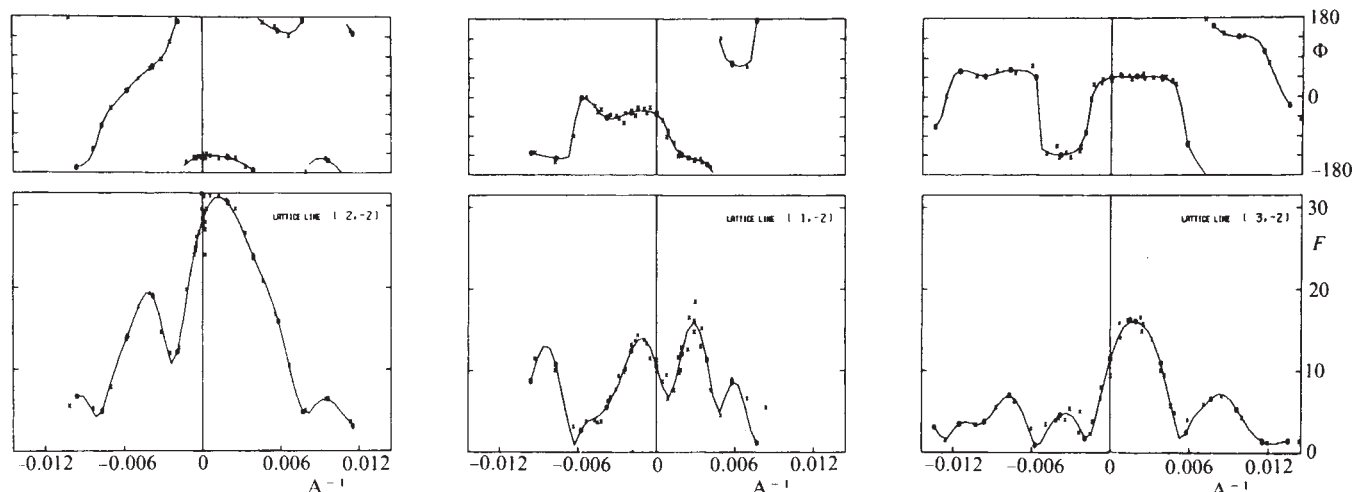


Fig. 3 Plots of observed Fourier transform data along three of the stronger lattice lines. In each pair of plots the lower one represents the amplitude and the upper one the phase. Observed points are plotted as crosses (x) and the equally spaced points interpolated by computer are plotted as circles (o) with continuous curves drawn between them.

thickness of about 600 Å estimated from the interference colour. This suggests that the section has shrunk considerably in the electron beam. Shrinkage of plastic sections during electron microscopy has been noted before^{5,6} and a detailed study⁶ showed that Araldite sections reduced in thickness by about 50%. Shrinkage of the M-band sections would result in the closing up of the M-bridge levels. A comparison of features common to the reconstruction from the oblique section³ with this map also suggests a shrinkage to 50% of the true value.

The final three-dimensional reconstruction was built up from two-dimensional density maps computed at axial spacings of 20 Å nominal, which would correspond to about 40 Å in the original muscle. One such map (Fig. 4b) not located at the centre of the reconstruction, contains clear 2-fold rotation axes

seen as mirror lines along directions of the hexagonal lattice connecting next-but-one neighbouring thick filaments. The bipolar nature of the thick filament⁷ implies that this represents the centre of the M-band (that is, the M1 bridge level). Examples of maps at equal separations (about 80 Å in the muscle) on either side of the M1 plane are shown in Fig. 4a and c. The same 2-fold symmetry relating these maps (and others not shown) is striking; there can be little doubt that the local symmetry of the M-band is described by the dihedral point group 32, and that the layer as a whole has space group P321.

Figure 5 shows two stereo pairs of the M-band reconstruction viewed from opposite sides. The M-band was not centrally located in the plastic section, and M1 lies deep in the model viewed as in Fig. 5a with M4 and the M6 region above it. In

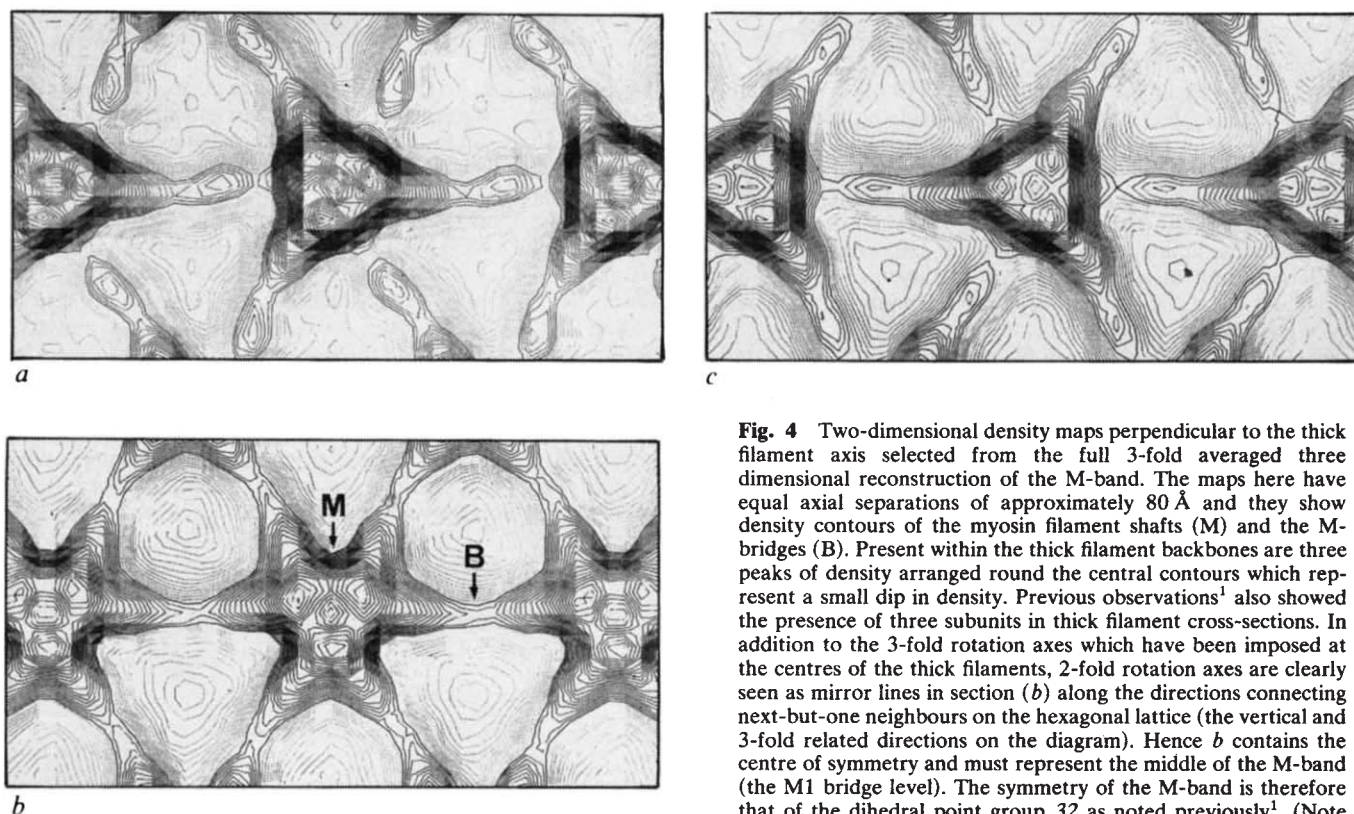


Fig. 4 Two-dimensional density maps perpendicular to the thick filament axis selected from the full 3-fold averaged three dimensional reconstruction of the M-band. The maps here have equal axial separations of approximately 80 Å and they show density contours of the myosin filament shafts (M) and the M-bridges (B). Present within the thick filament backbones are three peaks of density arranged round the central contours which represent a small dip in density. Previous observations¹ also showed the presence of three subunits in thick filament cross-sections. In addition to the 3-fold rotation axes which have been imposed at the centres of the thick filaments, 2-fold rotation axes are clearly seen as mirror lines in section (b) along the directions connecting next-but-one neighbours on the hexagonal lattice (the vertical and 3-fold related directions on the diagram). Hence b contains the centre of symmetry and must represent the middle of the M-band (the M1 bridge level). The symmetry of the M-band is therefore that of the dihedral point group 32 as noted previously¹. (Note that the density map in b was not located at the middle of the plastic section or of the three-dimensional reconstruction.)

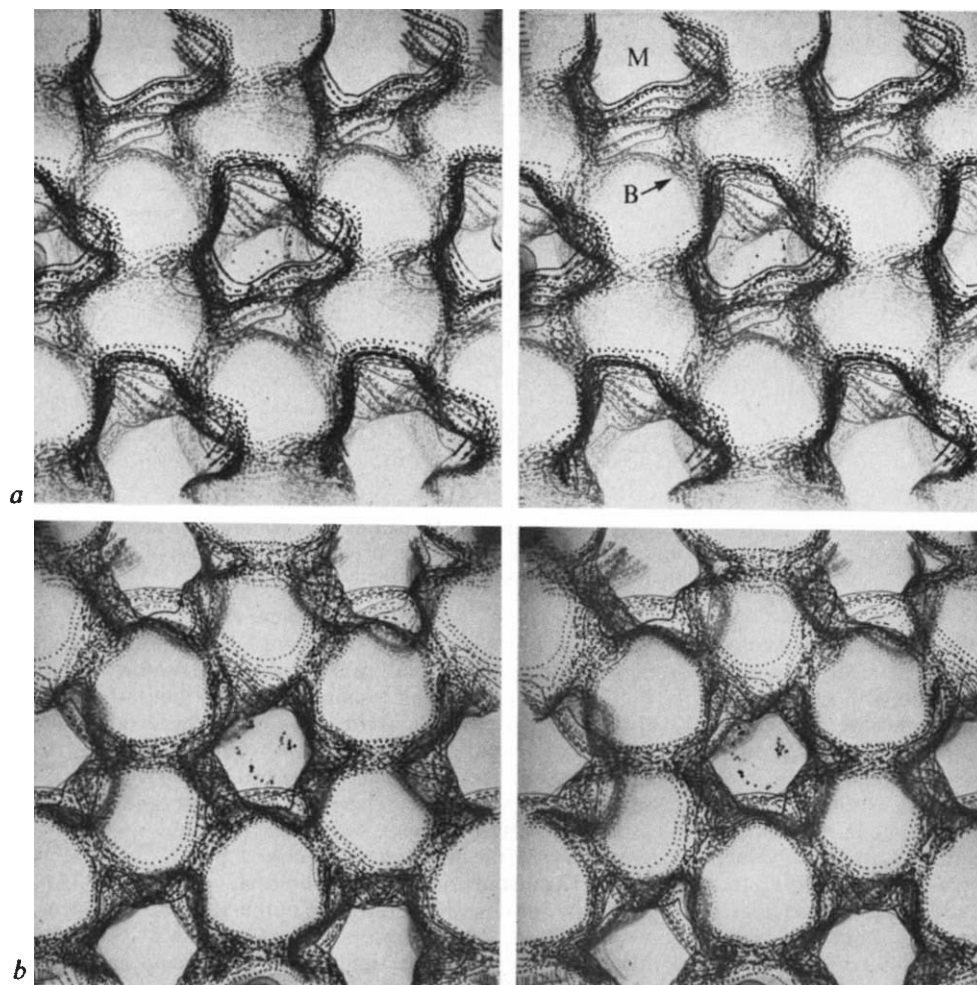


Fig. 5 Two stereo pairs of a Perspex model of the full three-dimensional reconstruction of the M-band viewed from 'above' (a) and 'below' (b). For clarity only three contour levels are shown. The dotted lines represent the lowest density, and the dashed and continuous lines represent respectively higher densities. Located in the centre of the images is a myosin thick filament (M). The lower part of the stereo view a shows the M-bridges (B) emanating from the thick filaments while the upper part shows the M6 region. The three points marked in the core of the central thick filament represent peaks of density. In b the peaks are seen to spiral through the M-band in an anticlockwise fashion viewed from the centre of the M-band. The stereo views clearly show 2-fold rotation axes in the M1 plane through next nearest thick filaments.

the opposite view shown in Fig. 5b M4' lies at the top surface. The 32 symmetry of the M-band is clear in both stereo pairs. In Fig. 5b, three dots marked in the core of the central thick filament represent peaks of density and their mean separation is about 75 Å relative to the 350 Å filament spacing. The peaks spiral through the M-band in an anticlockwise manner as viewed from the centre of the M-band. However, at the low resolution of the reconstruction, this spiralling is also consistent with parallel rods of density overlapping at M1 as suggested earlier¹. The hand of the whole M-band structure was confirmed independently using serial sections.

The outer profile of the thick filament is strongly modulated, giving the effect of ridges running along the filament. There are two sets of three ridges, one set on each side of the M1 level. The two sets are azimuthally out of register and overlap at the M1 level, giving the profile there a strongly six-lobed appearance (Fig. 4b). Moving from M1, one or other set quickly terminates, producing an overall triangular shape (Fig. 4a, c). These ridges run with a slight twist right up through the M6 region (Fig. 5a) and appear to terminate there³. The M-bridge density links to these ridges. It is not clear at this resolution whether to ascribe the density in the ridges to myosin tails, to M-bridges, to other M-band proteins or to some combination of these.

The three-dimensional reconstruction shows M-bridge density clearly but does not resolve the different axial levels of the M-band expected from the M-bridge stripes seen in longitudinal sections (Fig. 1). This may be because the plane of sectioning was not exactly transverse or may have wandered. Also the M-band itself may not have been exactly planar, since examination of longitudinal sections shows that the M-band may be slightly curved within a myofibril. These effects would lead

to axial smearing of the M-bridge density and consequent lack of axial resolution. Additionally, shrinkage normal to the section must close up the three M-bridge levels and make them less easy to resolve. A study of the shrinkage behaviour of plastic sections in the electron microscope is under way and the results will be reported elsewhere.

The application of three-dimensional reconstruction methods to plastic sections has produced a more quantitative and objective picture of the M-band than was previously available. The fish muscle M-band is established to have 32 symmetry and it is likely the thick filament as a whole has the same symmetry. Despite the factors limiting the resolution of the M-bridges, the axial details in the thick filaments are displayed more clearly here than could be seen directly, even in the ultrathin (200 Å) sections used previously¹. Further interpretation of the M-band must depend on specific labelling techniques. However, taken in conjunction with results from oblique reconstruction³, these maps provide constraints which future models of the M-band must satisfy.

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Three-dimensional reconstruction from a single oblique section of fish muscle M-band

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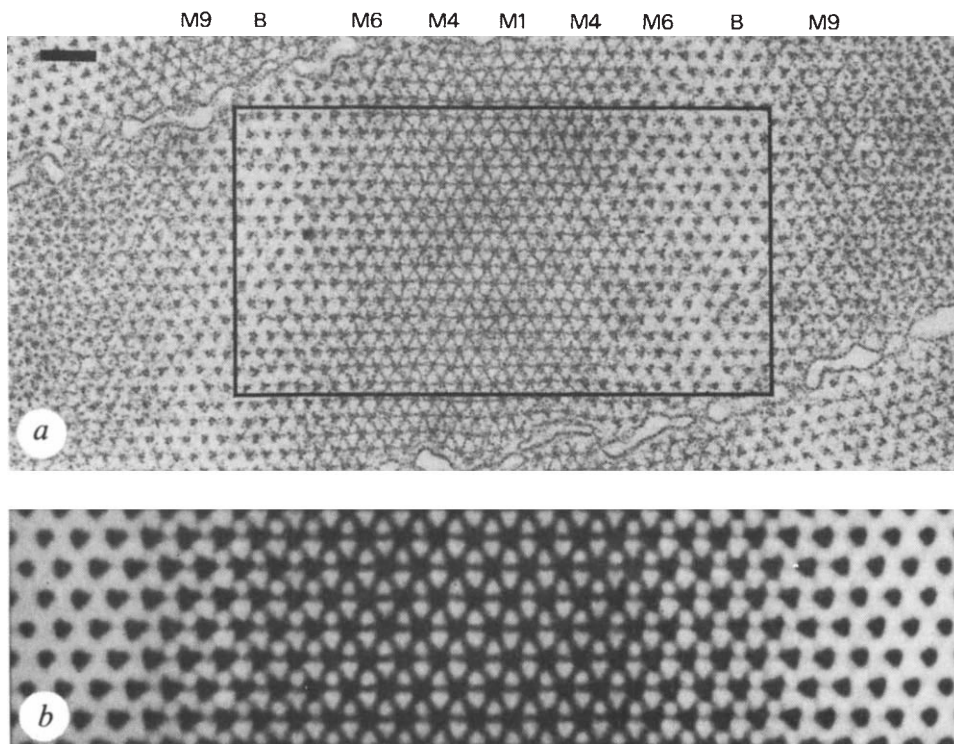
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A new method of three-dimensional image reconstruction from electron micrographs is presented here, which enables a three-dimensional image to be produced from a single oblique section of a two- or three-dimensional crystal. The method, which is most powerful when the section is thin and the angle of obliquity small, has several potential advantages over the conventional method of reconstruction¹ using tilted views of the section. In particular the accumulated electron dose on the specimen is much smaller and the reconstruction is not affected by changes in the thickness of the section during exposure to the electron beam. The method involves the solution of a generally almost singular set of linear projection equations², relating through the sectioning geometry the three-dimensional crystal density to the two-dimensional projected image density. This can be achieved most conveniently by linear least squares filtering³. An application of the method to determine the structure of the M-band of fish muscle is described. The resulting map agrees well with that produced by the more conventional approach involving tilted views⁴.

It is easiest both to visualize and to apply the method when the crystal is two-dimensional and when the direction of maximum slope of the section is normal to one of the cell edges of the crystal. Accordingly it is that situation which will be described and used here, although there should be no difficulty in working with a general oblique section of a three-dimensional crystal, except that the computation would be more complicated.

Fig. 1 *a*, Electron micrograph of a thin oblique, though approximately transverse, section of roach muscle M-band, prepared as described in ref. 7. The positively stained protein appears black and the thick filament profiles form an approximately hexagonal lattice. The direction of maximum slope lies left-right so that starting from the centre of the image one sees in both directions successively the M1, M4, M6, bare (B) and M9 regions of the M-band⁶. Scale bar, 100 nm. *b*, Strip image formed by one-dimensional averaging of the boxed area in *a*, normal to the direction of slope of the oblique section. The averaging by computer is performed directly on the image density, using bilinear interpolation⁹ to correct for small distortions in the thick filament lattice and correlation to get the best alignment of rows of unit cells before averaging. The result is a strip image one cell wide, which has here been extended by repetition to an area four cells wide, so that the pattern can be more clearly seen. The strip image is sampled every 2.2 nm at equivalent points within each cell, so simplifying the subsequent reconstruction. The distorted hexagonal lattice of thick filament profiles is thus made perfectly hexagonal and is represented most conveniently in the computer by a centred rectangular cell of size $a \times \sqrt{3}a$, where $a = 35$ nm is the mean separation of thick filaments. Every rectangular cell therefore has a thick filament at each corner and one in the centre. The features around each thick filament profile show 3-fold symmetry and the strip image as a whole has a vertical mirror plane at its centre.



With the above assumptions, the section contains rows of equivalent unit cells of the crystal (Fig. 1*a*), which can be averaged normal to the direction of maximum slope to give a 'strip image' one cell wide (Fig. 1*b*). Each cell of the strip image gives a projected superposition of a different set of levels from the full unit cell of the crystal (Fig. 2). As a consequence of the restrictive assumptions made about the sectioning geometry, the two-dimensional problem has been factorized, allowing one-dimensional averaging to give an improved signal-to-noise ratio in the strip image, followed by a simple one-dimensional reconstruction. For a general sectioning geometry this factorization would not be possible and the averaging and reconstruction could not be so separated.

The matrix of coefficients P_{ij} of the projection equations, $\sigma_i = \sum_j P_{ij} \rho_j$ (see Fig. 2) depends on the geometry of the crystal and of the section. In particular it depends on the rise per cell of the section and its thickness and these parameters must be known before the equations can be set up and solved. A choice must also be made of the pixel thickness and of the overall thickness of the specimen to be reconstructed. In general the projection equations comprise a rectangular set, with more observations σ_i than unknowns ρ_j , so that the system is most easily solved by least squares. It is nevertheless likely that the equations will be poorly conditioned, in that certain combinations of pixel weights ρ_j will be represented hardly at all in the projection data σ_i and will not therefore be recoverable without unacceptable noise amplification, as has been previously discussed for reconstruction from tilted views^{2,5}. It is therefore necessary to solve the projection equations by a least-squares filtering procedure³, in which only the best determined combinations of pixel weights ρ_j are recovered. This can be achieved by computing the eigensystem of the normal equations and recovering only those eigenvectors with eigenvalue λ such that $\lambda/\lambda_{\max}$ is greater than some specified filter level. The choice of filter level depends on the noise in the data and here a value of 0.01 was found to be appropriate.

For the M-band the sectioning geometry relative to the native muscle can be established approximately by inspection of the micrograph, since the separation of M9-M9' along the thick

Fig. 2 Geometry of oblique sectioning of a two-dimensional crystalline layer one unit cell thick. If it is assumed that the direction of maximum slope is normal to one of the cell edges, then the description of the geometry is simplified (see text). Each unit cell is considered to be divided up into a three-dimensional array of pixels of density ρ_j . The oblique section then cuts out a gradually changing set of pixels from equivalent columns of density in the various unit cells, as indicated by the stippled areas. When the oblique section is viewed in the electron microscope the resulting image will represent the projected densities σ_i , corresponding to summing the subsets of pixels cut out by the section. The unknown densities ρ_j in the specimen are thus related to the measured density values σ_i by linear projection equations of the form $\sigma_i = \sum_j P_{ij} \rho_j$, where $P_{ij} = 0$ if pixel ρ_j does not lie within the section in column i , $P_{ij} = 1$ if it does and P_{ij} will be a positive fraction if the pixel lies across the bounding surface of the section. For the simplified geometry considered, the σ_i will arise from equivalent points in each cell of the strip image and the different columns of density through the specimen can be reconstructed independently of each other. There will be one set of projection equations for each set of equivalent columns of density. Strictly speaking the oblique section ought to be tilted in the microscope to an angle equal to the sectioning angle before taking the picture. However, for small sectioning angles and thin sections the effect is not important, as can be seen in the strip image in Fig. 1b in which the thick filament profiles show no discernible elongation in the sectioning area.

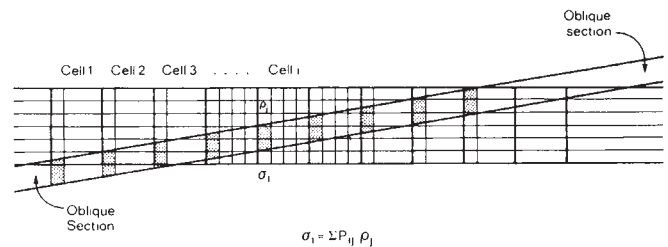
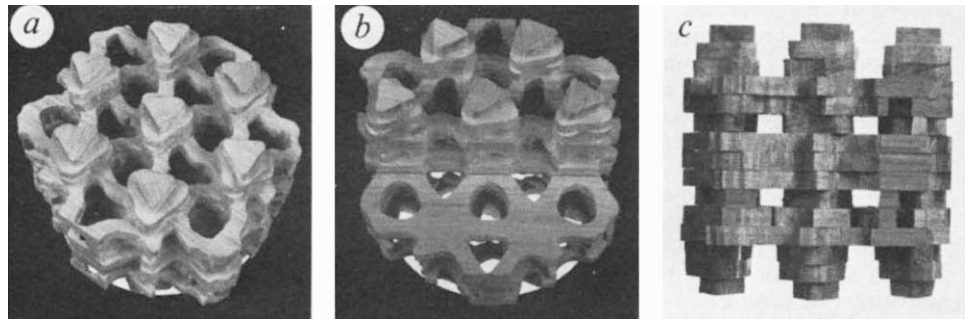


Fig. 3 A balsa wood model of the averaged reconstruction made from the strip image in Fig. 1b. Each section represents 6 nm and a total thickness of 90 nm is shown. a, The whole model consisting of a central thick filament surrounded by its six nearest neighbours. b, One-quarter of the model has been cut away to show the internal details. c, A side view along the direction of the hexagonal cell edge in which the filaments overlap. The 2-fold axis normal to the thick filament lies in the section revealed in the cut-away map b, which can be seen to have mirror lines through next nearest filaments. The absolute hand of the structure cannot be established from a single oblique section, so the handedness of the model was chosen to agree with that established for the reconstruction from tilted views⁴.



filament is known⁶ to be about 138 nm and in the oblique section (Fig. 1a) this rise takes place in about 38 thick filament spacings. Thus the section rise per filament or per unit cell in the strip image (Fig. 1b) is about 3.6 nm. The bare region between M6 and M9 is clearly visible but would not be seen if the section were thicker than about 30 nm. With these values of rise per cell and thickness, features should persist in the strip image for about six or seven cells, which appears to be the case here. The values of rise per cell and section thickness can be refined by setting up and solving the projection equations for a range of different values of the two parameters and taking those that give the smallest least squares residual as the best estimates. In this case the best values of rise per cell and section thickness were 3.62 nm and 27 nm respectively. This corresponds to a section angle of 5.9° to the strictly transverse for a thick filament spacing of 35 nm. A pixel depth of 6 nm was used and a total thickness of 102 nm reconstructed, giving 17 levels in all. However, only 12 of the 17 possible eigenfunctions satisfied the recoverability criterion $\lambda/\lambda_{\max} > 0.01$ (see above), giving an effective independent pixel depth in the reconstruction of $102/12 = 8.5$ nm. This is rather less than one-third of the section thickness, so that the reconstruction procedure has produced a map with markedly better depth discrimination than can be obtained simply by looking at the strip image.

The M-band has been shown^{4,7} to have 32 symmetry and it is clear that the strip image (Fig. 1b) has an overall vertical mirror plane at its centre and that the features around each thick filament profile are strongly 3-fold related. The map produced by the reconstruction procedures was therefore 3-fold and 2-fold averaged along appropriate directions to improve its signal-to-noise ratio. A model of this averaged map is shown in Fig. 3.

The reconstruction shows that the thick filaments are connected by strong fairly straight bridges (M1) at the centre of the M-band. About 22 nm away on either side the filaments are connected by curved bridges (M4, M4'). The thick filaments

have a rather uniform diameter throughout the M-region but become much thinner about 40 nm from the centre of the M-band as is also clearly seen in the strip image (Fig. 1b). This appearance could be due to ridges of extra material running along the thick filament, giving the filaments a strongly three-lobed cross-section throughout the M4–M6 region. These ridges may well be formed partly or wholly by myomesin, which seems to run throughout the central M-region but which probably does not contribute to the M1 or M4 bridges⁸. The curved bridges at M4, which may be formed by creatine kinase⁸, appear to start and end on these ridges.

The features seen in this oblique reconstruction agree well with those seen in the reconstruction from tilted views⁴. In particular, the shapes of the holes outlined by the thick filaments and M-bridges vary from section to section in the same way in the two maps. The principal differences are that in the oblique reconstruction the M4 bridges are more clearly visualized and the spacings along the filament direction are not affected by section collapse, because the computed geometry of the section is based on spacings close to those of the native muscle. Thus although this method of oblique reconstruction is intrinsically less powerful than that based on tilted views, it does nevertheless have some advantages and certainly gives improved depth discrimination compared with that obtained by direct viewing of oblique sections.

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Customers and contractors

Philip Gummett

Government and Research: The Rothschild Experiment in a Government Department.

By Maurice Kogan and Mary Henkel.

Heinemann Educational: 1983. Pp.196. £18.50, \$37.50.

THE Rothschild Report of 1971 presaged one of the biggest shake-ups ever administered to the organization of science in Britain. It may in time be seen as the high point of the sectoral (or departmentally centred) approach to British science policy, especially if the Government adopts the reforms proposed by Sir Ronald Mason* in November 1983; these would strengthen the Advisory Board for the Research Councils and require all government research (civil and defence) to be included in the debate over the Science Budget of the Department of Education and Science, thus increasing central coordination.

Under Rothschild, government departments were to assume greater responsibility for formulating policy for applied research, which would then be implemented by their contractors. These might be in in-house research establishments, research councils, or elsewhere. By the same token, these contractors were to lose control over their applied research programmes, a particularly painful blow for the three bodies involved, namely, the Agricultural (ARC), Medical (MRC) and Natural Environment (NERC) Research Councils. The implications of the new regime were, however, greater in some departments than others, being modelled on the then current practice of the Ministry of Defence, and not involving much change in organizational reforms already in train at the Department of Trade and Industry.

The only attempt so far at overall evaluation of these reforms has been the one conducted by the government itself (*Review of the Framework for Government Research and Development (Cmnd 5046)*; HMSO, 1979, Cmnd 7499); not surprisingly, it was short on analysis though useful as a summary of organizational developments. Kogan and Henkel's book, in contrast, is a detailed, thoroughly researched and methodologically path-breaking examination of how one part of the science-government system, the Department of Health and Social Security (DHSS) and the MRC, responded to Rothschild.

From 1974 the senior author worked as a consultant to DHSS on the operation of the post-Rothschild research management system. He and his research team enjoyed access to internal papers, attended over 200

departmental meetings, conducted over 200 interviews with key individuals and accompanied the Chief Scientist on some of his visits to external research units. The initiative of the DHSS in thus engaging Professor Kogan, and the encouragement

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Lord Rothschild — the report of 1971 "presaged one of the biggest shake-ups ever administered to the organization of science in Britain".

given to him to publish, are to be applauded. The high quality of the work should encourage more departments to engage researchers in "on-line" evaluation of organizational reforms and, in an ideal world, it would also mark the end of bland official reviews like Cmnd 7499.

The price for this exceptional degree of access might well have been a story lacking in colour. And, indeed, most of the characters are not fully identified. But in general this does not matter: it is usually enough to know that we are dealing with an under-secretary in a policy division, or a member of the Chief Medical Officer's staff. Thanks, however, to the encourage-

ment of Professor Arthur Buller, we do learn a lot about his own role when Chief Scientist at the DHSS, and this is all the more creditable because the authors make plain their disagreement with his view of science and its relationship with policy. We learn, for example, that he was less interested in cultivating the system for enunciating biomedical research policy (and thereby maintaining control over the funds transferred from the MRC) than in raising the quality of health services research (p.73); and that on his appointment in 1978 he determined to build up the scientific strength of the Department's research base, in part by strengthening the role of his own office in the commissioning of research at the expense of other parts of the machinery in which policymakers from DHSS divisions took the lead (p.86).

One cannot help wondering, though, what else could be said about how, in 1981, the MRC recaptured the funds lost to DHSS in 1972. For example, in addition to the point made above about the Chief Scientist's priorities, we also read, on p.73, that the MRC, in contrast to the Social Science Research Council (with which the Department also dealt), "enjoyed the confidence of a single, and supremely self-confident, profession strongly represented within" the DHSS. This sentence clearly alludes, in conformity with a theme well-documented in the book, to the institutional power of the biomedical research community and to its ability to impose its view that scientific development should be driven more by the internal norms of science than by social considerations. But does it also allude to the role of the Chief Medical Officer and his staff? One recalls here Sir Alec Merrison's tentative reference, before the House of Lords Select Committee on Science and Technology in April 1981, to the problems of fitting a Chief Scientist into a department which already had a powerful Chief Medical Officer. The role of the politicians in the DHSS is also largely passed over: it is unclear how far the attempt made from 1979 to cut spending on DHSS-funded research units derived from the political level (there being a General Election that year); and, again with reference to the recapture by the MRC of the transferred funds, what precisely lay behind the observation that MRC scientists "knew that they could beat the bureaucrats by appeals to the political system above their heads" (p.75)?

One other caveat: the organizational arrangements within the DHSS were extremely complex and perhaps required more careful description. In particular, the Research Liaison Groups (committees which brought together the policy divisions and scientific advisers to work out research programmes for specific policy areas) are introduced on p.12 as though they covered all aspects of the Department's work; on the next page we learn that many areas were

*Advisory Board for the Research Councils, *A Study of Commissioned Research: Report by Sir Ronald Mason*. Available from the Department of Education and Science, London.

not covered; only on p.77 are we told which areas were covered, having in passing (p.59) learnt that the rationale underlying these committees was to stimulate important but under-researched zones of action. It is clear enough when pieced together, but the task could have been easier.

These are trivial reservations, however, in comparison with the authors' achievements. For the book is not only a detailed review of post-Rothschild developments in the DHSS — a notable feat in itself, if one of limited appeal. It is also an original exercise in the combination of the approaches of the sociology of science and of public administration to the study of science policy. As such, it is a rich quarry of ideas on how to conduct such studies, and on the sheer complexity of the subject. It should be read not only by academic students of science policy and public administration, but by all who are concerned, in whatever way, with relations between science and government.

The book examines theoretically the extent to which science is steerable, or, in a different metaphor, permeable by social values. Drawing on the "finalization" school of sociology of science, the authors distinguish between different phases in the development of disciplines, arguing that disciplines are more steerable (though in different ways) when in the pre- and post-paradigmatic phases, but that in between, where work is beginning to crystallize around key theoretical models, the research programme is much more dictated by "internal" needs and is less susceptible to outside influence. They show how epistemological ideas must be linked with analyses of the institutional power of different sciences (contrast, for example, most medical research with most social science) in order to grasp the heterogeneity of science and the complexity of its relationships with policy. One might add, in the context of increasing interest in the evaluation of research, that these same considerations should urge caution in trying to apply blanket measures of research performance: the form, as well as the substance, of the products of different disciplines varies and demands great sensitivity in cross-disciplinary evaluations.

On the customer side of the picture, the authors show the variety of roles that exist in government, and the range of expectations that can be held of science. Officials concerned with policy-making, scientific advice, research management and research liaison have markedly different perspectives on science-government relations. Moreover, policymakers themselves are heterogeneous: some take a purely instrumental and short-term view of research; others, especially those with remits concerned more with policy analysis, are more interested in how research might help develop policy. Much emphasis is given to the need for, and complexity of, "brokerage" between departments and

research councils, and between policy and science. We also learn a good deal about the need for sustained, long-term commitment to the development of the customer role, a process not aided by the relatively rapid turnover of staff on that side of the customer-contractor relationship.

Not surprisingly, the authors conclude that no single or straightforward model of science policy is appropriate, and that the metaphors of customer and contractor are oversimple. The customer metaphor implies that government can be clear and authoritative in stating its objectives "before entering the marketplace to purchase knowledge" (p.164). The contractor metaphor likewise assumes a coherent, identifiable and independent scientific community, able to negotiate from strength. In practice, both sides of the relationship exhibit a multiplicity of goals, functions, epistemologies and power bases. The social steerability of science

depends, therefore, in a complex way on the effort put in by the customer, on the developmental state and institutional power of the scientific discipline concerned and on the level in the system at which one is working: micro-policy questions are easier to resolve than macro ones. Hence, finally, the conclusion that, at least in the broad field of social welfare, government should apply its energies to interaction with science rather than steering of science: it should be concerned with the impact and implementation of research, and with the slow, steady and sustained development of strong communities of researchers and policy makers, rather than with rationalistic or imperative planning. □

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East-West pairing

R.J. Elliott

Excitons.

Edited by E.I. Rashba and
M.D. Sturge.

North-Holland: 1983: Pp.860.
Dfl.425, \$181.

Excitons is one of the first (actually Volume 2) of a series of books entitled *Modern Problems in Condensed Matter Sciences*, to be edited by V. M. Agranovich and A. A. Maradudin, and published jointly in English by North-Holland and subsequently in Russian by the publishing house Nauka. Each volume has editors and contributors who, like the general editors, are drawn from the Soviet Union and from the West.

As we all know, communication with Soviet colleagues is not as easy as we would like, partly because of language differences and partly because of the restrictions on travel to and from the Soviet Union. It is therefore particularly useful to have a series of up-to-date reviews which come in this form. It will make it less easy for scientists in the West to overlook Russian work in related fields.

Excitons are particularly appropriate for such a joint review. One leg of the theoretical foundation of the subject is firmly planted in Russia, on the work of Frenkel, Pekar and others, and relates largely to ionic and organic materials. The other is based in the West on the work of Wannier, Mott and others, and refers most directly to semiconductors. Experimental work in both areas has been carried out in parallel in all countries; for example, the rich exciton spectrum of cuprous oxide was evaluated in detail by Gross in Leningrad and by Nikitine in Strasbourg, and similar parallels exist in the work on polariton

effects and on high-density excitons such as electron-hole droplets.

The book contains 18 chapters devoted to recent developments in this vigorous area of solid state physics and chemistry, their diversity displaying the remarkable wealth of the subject. Several refer to polariton (excitron) effects, which lead to spatial dispersion and non-local effects, which appear remarkable and unusual in classical optics. Another group of papers deals with the effects of perturbations on exciton spectra, either arising from applied electric and magnetic fields, or from impurities. Isolated impurities cause trapping and in a semiconductor alloy the large number of impurities can give rise to different and important effects. Multi-exciton complexes are also considered, ranging from the relatively simple bi-excitons in CuCl to the many particle electron-hole complexes found in some semiconductors. Finally, the book's coverage has been extended to cover excitons in more specialized materials, including strained molecular crystals, magnetic insulators, and photosynthetic and other biological systems. Vibrational excitons (bound phonon pairs due to molecular anharmonicity) are also included.

The quality of the articles is uniformly high. There is naturally some variation in approach, but this is inevitable in the treatment of such a diverse field. The editors have brought together so many aspects of exciton physics that all those with an interest in the subject are bound to find not only useful reviews of their own speciality, but interesting insights into related fields. This volume augurs well for the rest of the series — I hope that at least some libraries will be able to afford it. □

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Lead in history and history in lead

Josef Eisinger

Lead and Lead Poisoning in Antiquity.

By Jerome O. Nriagu.

Wiley: 1983. Pp.437. £52.20, \$73.10.

AMONG the technological arts discovered by our ancestors, none has played a larger role in shaping our civilization than metallurgy; and because the common minerals of lead melt at low temperatures, lead was the first metal to be refined. Lead may also have given rise to our oldest chemical industry, the production of white lead (ceruse), and is responsible for our oldest occupational disease.

The oldest lead object found by archaeologists is a string of beads worn in Anatolia some 8,000 years ago, and its use in jewellery suggests that this was a time when lead was still new and rare. Because of lead's malleability, corrosion resistance and other desirable qualities, human ingenuity devised many other uses for the metal and it attained great commercial importance in the classical world. The lead mines of Cartagena in southern Spain were exploited by Carthage before the area was conquered by Rome, and were worked by 40,000 miners who left a legacy of more than 10^7 tons of slag. In antiquity, lead was employed in coinage, glass, medicines, warfare (as sling bullets and in molten form during sieges), ship building, cosmetics (various oxides as eye shadows and so on), glazes for pottery, pigments, masonry (as mortar) and, of course, in "plumbing".

These are just a few of the myriad facts which Dr Nriagu has collected in his book, along with poetic and literary references to lead, arcane lead lore and the role of lead in alchemical theory. As a geologist and geochemist, the author is on familiar ground when dealing with the lead resources of the ancient world, including China and India, and with the contemporary methods of prospecting, mining and production. But he covers virtually all aspects of the metal's ancient history and has assembled an impressive bibliography from diverse disciplines which will be of great value.

The presentation of this encyclopaedic work is unfortunately not commensurate with its scope, however. The problem of organizing so much information into a readable text has not always been solved successfully: too many facts and quotations are offered without the necessary background or critical comment, and an unusually sloppy proof-reading job has left

an abundance of annoying misspellings.

No aspect of the history of lead discussed by Dr Nriagu is likely to provoke as much interest and controversy as the prevalence of chronic lead disease in antiquity and his suggestion that it played an important part in the decline of the Roman Empire. This thesis was first advanced by Gilfillan in 1965 and has, ever since, irritated historians and classicists who are familiar with the complexities of that event. Gilfillan's historical model nevertheless has great popular appeal, and questions about it are raised in almost every discussion of the history of lead poisoning.

There exists, indeed, a great deal of literary, experimental and, lately, an increasing amount of archaeological evidence that many Romans were exposed to dangerous levels of lead. The most

societies and $30\text{--}50\text{ }\mu\text{gd}^{-1}$ for the modern American.

Dr Nriagu raises the question why, in a society in which occupational plumbism, particularly among miners, was rampant, the connection between lead ingestion and disease remained undiscovered. Quite apart from the fact that miners were generally slaves or prisoners of war, beyond humanitarian concern or the care of physicians, the notion of a specific cause for a chronic disease, which we take for granted, was quite foreign to Graeco-Roman medicine. Galen and other contemporary physicians saw disease, including lead colic, as the result of an imbalance of the humours, and this view prevailed until some 1,500 years later when Paracelsus and his followers insisted that disease had specific chemical causes. The

aetiology of the colic epidemics which were caused by the consumption of leaded wines and which persisted for many centuries was, in fact, not identified until 1696 (see *Med. Hist.* 26, 279; 1982).

But while a strong case can be made for widespread lead disease in the Roman Empire and excessive drinking at the courts of some emperors, does it justify Gilfillan's hypothesis?

Dr Nriagu considers that the gluttony and other excesses of the Julio-Claudian and Flavian emperors were lead-related, although this is difficult to reconcile with the loss of appetite and constipation which are among the prominent symptoms of chronic plumbism. And did not the Empire attain its greatest wealth, power and extent under Trajan and other sober and effective emperors, who succeeded these sodden miscreants? It has on the other hand been suggested that gradual

depopulation contributed to the failure of the Western Empire to defend itself against the invasions of the fifth century, and lead-induced infecundity may indeed have played a part in this. But the disappearance of the patriarchal families in the first century can and has been explained otherwise, and may indeed have strengthened the state by introducing new blood into the most enduring political system which the West has evolved.

In the final analysis the evidence which has been marshalled in support of Gilfillan's hypothesis is, at best, anecdotal and occasionally spurious. There is little doubt that countless Roman citizens suffered and died from plumbism, but the link between that health disaster and the fall of the Roman Empire not only remains unproven but is probably unprovable. □

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Smelting lead in the sixteenth century. The illustration comes from one of the works of Georgius Agricola (1494–1555), the German mineralogist who was the first to attempt to impose scientific order on the hitherto unsystematized knowledge of miners. (Reproduced from *Gmelins Handbuch der anorganischen Chemie*, Vol.47, Pt A1; Verlag Chemie, 1973.)

important exposure resulted from the unhappy custom of preserving and sweetening wine by the addition of a syrup (*sapa*), which was prepared by simmering grape juice (must) in a lead kettle. It has been shown that *sapa* prepared according to any of several first-century recipes contained about 1gPb l^{-1} , and when added to wine in the recommended proportion would produce chronic lead disease. Without *sapa*, or other preservatives such as resin which was, and is, preferred by the Greeks, the extensive wine trade of the Roman Empire would have been impossible.

Even those Romans who eschewed leaded wines ingested *sapa*, for it was a common cooking ingredient, and saturnine cosmetics and medicines were widely used. Dr Nriagu estimates that the rate of lead absorption ranged from $15\mu\text{gd}^{-1}$ for slaves to $250\mu\text{gd}^{-1}$ for aristocrats, compared to $2\mu\text{gd}^{-1}$ in pre-technological

Chandrasekhar on Eddington

Fred Hoyle

Eddington: The Most Distinguished Astrophysicist of his Time.

By S. Chandrasekhar

Cambridge University Press: 1983. Pp. 64.
£7.50, \$12.50.

ARTHUR Stanley Eddington was born on 28 December, 1882. Two centenary lectures were given in his memory, and the Master and Council of Trinity College, Cambridge, made the happy decision of asking Professor Chandrasekhar to deliver them. To this assignment he brought to bear all the care and scholarship which has attended his work down the years. The first of his lectures bears the same name as the book itself; the second is entitled "Eddington: The Expositor and the Exponent of General Relativity".

The first lecture has five sections: parts I and II are biographical, III is concerned with Eddington's early researches on stellar motions, IV is on stellar structure, and V is an amusing summary of Eddington's relations with his contemporaries, relations both friendly and stormy. The work on stellar structure, the internal constitution of the stars as Eddington preferred to call it, was of such massive proportions that I must pass it by except for the following brief sketch. Of the factors on which the luminous output of a star depends, Eddington showed that by far the most important is its mass. Given the mass it was possible to predict, without any knowledge of the source of a star's energy, what the luminous output must be, and to do so with a degree of accuracy that was considered remarkable in the 1920s.

Even within the short space available in a lecture, Professor Chandrasekhar brings out the remarkable degree to which Eddington was self-contained in his work, less influenced by others immediately around him than is usual for a scientist and reminding us of Peer Gynt's motto: To thyself be sufficient.

The second lecture has nine sections. Part I is a brief description of the origins of Einstein's theory, II is concerned with Eddington's early enthusiasm for the theory and his part in organizing the eclipse expeditions to test the predicted bending of light past the Sun, and III contains Professor Chandrasekhar's own recollections about this episode in Eddington's life. I was amused to find this problem: "A affirms that B denies that C declares that D is a liar" rearing its head again, a problem which last tormented me in the year 1936 when I attended Eddington's lecture course, "The Combination of Observations". There were turbulent arguments among us students about it, and frankly speaking I never could see that a

determination of the probability that D spoke the truth had a solution. I am glad even after these many years to find Professor Chandrasekhar slipping in the extra information that D really made a statement. Possibly Eddington put in this condition himself, but it was hard to judge from his lectures; unlike the beautiful clarity of his writing, Eddington's lectures were a quagmire through which we heaved ourselves as best we could.

Part IV of the second lecture is an appraisal in glowing terms of Eddington's splendid book, *Mathematical Theory of Relativity*. Like Professor Chandrasekhar, I too continue to use it. In Part V we have a brief exposition of Eddington's attempt at a unified theory of gravitation and electromagnetism. Then after a light relief in VI, with much quoting of poetry, we come to what Professor Chandrasekhar calls the unhappy development of Eddington's ideas, to what he himself referred to as fundamental theory, an attempted fusion of physics and cosmology, which Professor Chandrasekhar describes with plenty of supporting quotations as a transition from sureness to cocksureness.

Where, one can ask, does praiseworthy sureness end and unpraiseworthy cocksureness begin? Since there are plenty of examples of important work being ignored

for many years, it is hardly a satisfactory answer that the judgement lies in the reactions of a man's contemporaries. The issue is better related to fact. Where facts are many or are strong, it is possible to be sure. Where they are few, and are subject to a variety of interpretations, sureness becomes cocksureness, which unfortunately one must agree was the situation in Eddington's later work.

Nevertheless, one word of defence! Is Eddington's cosmology really negated because of the discovery of primordial helium and the microwave background? With a non-zero cosmical constant, one can have an initial big-bang that is adjusted by a special choice of parameters to pass asymptotically close to the Einstein static state, and with this special choice Eddington could have defended himself, as indeed Lemaître did. Discovery of the existence of primordial helium and the microwave background date from 1965. Is it not a little ominous for those who are so cocksure about their ideas in cosmology today that the factual evidence for their position has remained nearly static over almost two decades? □

Sir Fred Hoyle was an undergraduate student of Arthur Eddington's at the University of Cambridge in 1935-1936.

Son of John Paul

G.D. Clarke

Culture of Animal Cells: A Manual of Basic Technique.

By R. Ian Freshney.

Alan R. Liss/Wiley: 1983. Pp. 295.

\$49.50, £33.

THE washing of glassware, media making and cell production may seem generations away from gene cloning, monoclonal antibodies and fluorescent-activated cell sorting. They are not of course; but in large laboratories they are a central service and, if efficiently carried out, those who perform them may not be readily identified in corridors or canteens. In a decade of rapid advances, especially in molecular genetics, such services may be undervalued, but never by one used to a less-favoured environment.

The major part of Ian Freshney's book covers the setting up of a cell culture facility and the routine methods necessary to its function. The author then gives brief protocols for the more specialized techniques, referring always to the original publications. The bibliography is excellent and the cross-referenced list of equipment and reagents with addresses of manufacturers (in Europe and North America) will prove useful to many. The employment of trained staff, or advice from experienced colleagues, is preferable to book learning for those setting up a

culture unit from scratch; but this book, with its large format, *Scientific American* lucidity, and generous use of diagrams, will be an essential adjunct.

The protocols, which have been taken direct from the Methods Book of a versatile laboratory, must be almost foolproof. Nevertheless, novices looking only for one particular procedure would be advised to spend some hours on a general browse. History records that many wrinkles and pitfalls are only obvious after expensive and time-consuming experience; Ian Freshney mentions them all somewhere but they could be constantly repeated.

Common sense suggests that the sections on organization and routine procedures will be of greatest use in developing countries, where budgets are tight and labour cheap. So a short appendix on alternative technology might have been included, while the absence of reference to probabilistic events in the sections on cell synchrony and physical cell separation could lead biochemists into an inappropriate use of cell populations. But these are small factors in a book invaluable in a wide range of rapidly expanding fields. The simple presentation of established routines, and introduction to the invaluable serum-free media of Ham and Sato, and to modern molecular techniques, make this a worthy competitor for Professor Paul's classic of 1959-1975. □

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General

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Computers in chromatography

Richard Patience gives a personal view of how to tackle the growing array of computers being used in chromatography.

How many chemists and biochemists, when faced with a computerized chromatography system, know which button to press and when to press it, but have no idea how the system works? My own first encounter with such a system — a DEC PDPB/e laboratory computer attached to a capillary gas chromatograph/mass spectrometer (GC/MS) — only served to give me a firm conviction that it was all far too complex and that I would never learn to use it. Of course, in time, I did and, what is more, became quite at home with it. But it is one thing to know what to do with a computer keyboard and quite another to know why you do it!

In the mid 1970s, when I first started working with computerized chromatography, laboratory computers were quite rare — and quite large — and generally found only on more sophisticated and expensive equipment, like GC/MS. Temperature and solvent gradients in GC and liquid chromatography (LC) respectively were set up manually. The Pye 104, the Perkin Elmer F17 and the Varian 2700 GCs were all operated in this way, whilst the major LC company, Waters, had the M660 gradient analysis programmer, which has only recently been superseded by the microprocessor-driven M680. 'Data collection' was by chart recorder.

Shortly after I embarked on my research career, there were two major developments. First, in gradient formation, microprocessors began to replace manual control, particularly in GC. Microprocessor-controlled gradients were set up from a small keyboard and parameters such as gradient slope were stored in the memory. Automatic cooling at the end of the run meant that the whole cycle could be pre-programmed and initiated by a single key, but unfortunately you still had to inject the sample.

The second improvement came with the arrival of integrators to calculate peak areas for quantitation. This was a real advantage — both in terms of time and accuracy — even though some early models produced only a numerical strip print out and no chromatogram.

One of the main advantages claimed for microprocessors was an improvement in reproducibility of repeat analyses, in other words an increase in retention times. I have no reason to dispute that, but in my opinion there were, and still are, two disadvantages to microprocessors. The problem with many systems was that, unlike manual units, once started the programme could not be altered without aborting the

whole run. This gives a degree of inflexibility that is fine for routine analyses but can frequently be inconvenient when dealing with less well defined samples. What is more, gradient controllers and integrators originally had to be programmed and started separately. The poor user needed three or four hands — one to inject (two for the less steady-handed), one to initiate the gradient programmer and one to start the integrator.

Now, of course, all this has changed and often the whole analysis sequence is completely automatic. The computer boom has reached even HPLC — slower to benefit than GC but rapidly catching up. So, faced with the present array of chromatographic systems, how does the practical biochemist or chemist decide which best suits his or her needs? The mechanical aspects are relatively easy; from practical experience of dismantling pumps and setting up equipment it is possible to assess the mechanics of an HPLC system. But computers — particularly software — are much harder to evaluate without actually using them. If this be the case, then where does one start?

Choosing a system

One tactic is to begin with an assessment of the facilities available from the present generation of computers. Fig. 1 shows a stylized configuration of a hypothetical HPLC system. The controller can be programmed to initiate and control a number of events at preselected times. Furthermore when remote computers or terminals are linked up, the system can be controlled

remotely and data can be generated and sent to distant units.

The range of functions offered by individual manufacturers varies considerably, of course. More sophisticated systems include the Varian Vista 402, the Spectra Physics Labnet, Waters' M721 and associated units, the LDC CCM system, Perkin Elmer's Series 4 + Chromatographics 2, Pye Unicam's 4850, Hewlett Packard's 1090 + HP85, Dupont's 8800 and Sentinel, and Gilson and ACS, which use Apple IIe computers.

Why, though, should such sophistication interest a typical chromatographer? One advantage of microprocessor-based systems has already been mentioned — increased reproducibility of retention times of repeat analyses. However, to me the main advantage lies in the ability to co-ordinate different units and events from a single keyboard. Gone is the need for three hands to start a run; with fully automated instruments one finger may be enough for dozens of analyses. Of course there are always disadvantages. One of these is cost — around £7,000 to £10,000 at present on top of the HPLC cost. Another is simply the level of complexity of the system itself, which can be confusing for non-specialists, and inevitably introduces a degree of redundancy to the system. Nevertheless, I am sure that the use of computers in chromatography will continue to increase, particularly as they become cheaper. □

Richard Patience is in the department of chemical endocrinology at Saint Bartholomew's Hospital, London.

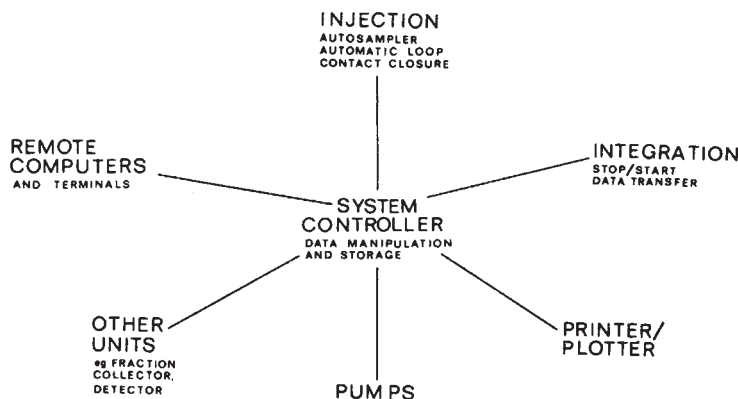


Fig. 1. Stylized configuration of a computerized HPLC system. The controller regulates; (1) injection, which may be by autosampler, automatic loop (user loads loop, controller injects it) or contact closure (user loads and injects, which closes electrical contact and starts controller program); (2) pumps: gradient formation preprogrammed; (3) integration: stop/start or integration and transfer of data to the controller for manipulation, storage on disk or tape, or plotting; (4) printer/plotter; (5) other units, such as a fraction collector or detector; (6) remote computers or terminals.

High performance liquid chromatography

● HPLC Technology has launched a range of HPLC column block heaters. The heaters operate in the temperature range ambient to 99°C and reproduce temperatures with an accuracy of $\pm 0.5^\circ\text{C}$; their temperature rise time from ambient to 60°C is 10 minutes. Columns of various lengths (with or without pre-columns) can be used. Heaters are available for larger diameter columns as well as for heating the injection system.

Circle No. 100 on Reader Service Card.

● Perkin-Elmer has introduced several new HPLC columns. The 3×3 columns, so-called because they are 3 cm long and use 3- μm packing materials, offer reduced analysis times and lower solvent consumption. The short column length eliminates high back-pressures. The columns can be used with most liquid chromatography systems, but operate best with Perkin-Elmer equipment. The combination of the Perkin-Elmer series 4 solvent delivery system, the LC-85B detector with a 1.4- μl flowcell and 3×3 columns provides the fastest analyses.

Circle No. 101 on Reader Service Card.

● Bio-Rad's HPLC column heater will produce temperatures between ambient +5°C and 150°C with accuracy of $\pm 2^\circ\text{C}$, while operating in environments with temperatures between -5°C and 50°C. A thermostat set to turn off power automatically at 180°C provides over-temperature protection. After a standard warm up period, the column heater will maintain temperatures with a stability of $\pm 0.1^\circ\text{C}$. The heater has a dual column capacity, and can accommodate columns from 5 to 30 cm in length and from 1/4 to 1/2 inch in diameter. A 'solvent preheat block' is also available as an accessory.

Circle No. 102 on Reader Service Card.

● A series of prepacked HPLC columns of high-pressure hydroxyapatite has been introduced by Bio-Rad. The Bio-Gel HPHT system has a standard column, which is 100 × 7.8 mm and has a capacity of 100 mg for protein or 5 mg for DNA. Separations can be concluded within 30 minutes. Any technique using standard hydroxyapatite protocol can be applied to the high-pressure system. The columns can be used to purify monoclonal antibodies from mouse ascites fluid and to purify plasmids. They are either available individually or as a system which incorporates the use of a sacrificial guard column.

Circle No. 103 on Reader Service Card.

● Gelman Sciences has introduced a miniature disposable membrane filter unit designed for HPLC users. The Acro LC13 has a 0.2, 0.45 or 1.0- μm , 13-mm diameter fluoropolymer membrane filter sealed into a polypropylene housing. This reduces liquid sample loss to 0.014 ml. The new fluoropolymer membrane is naturally wetted by aqueous samples whilst retaining chemical compatibility with solvents such as methanol, chloroform, *N*-heptane, methylene chloride and acetonitrile.

Circle No. 104 on Reader Service Card.

● A new alkylphenone HPLC internal standard kit is now available from Pierce. Alkylphenones are a family of neutral compounds. Neither solvent modifiers nor acidic nor basic buffers will affect their retention volume. Due to their wide solubility range, both polar and non-polar solvent systems can be used. Nanogram quantities are detectable with a common UV detector. Each kit contains one gram each of nine alkylphenones.

Circle No. 105 on Reader Service Card.

● A new HPLC pumping system has been introduced by Knauer. It has an electronically-controlled motor, two sapphire pistons and two cartridge-type ruby ball check valves. Its unusual cam design gives correct precompression and will compensate the pressure feedback electronically for liquid compressibility. Changing from micro- to macro-chromatography is achieved by exchanging pump heads. The unit can be interfaced with most commercial controllers or with its companion microprocessor programmer models 50A and B.

Circle No. 106 on Reader Service Card.

● A new carbohydrate HPLC column is now available from IBM Instruments. The new column is packed with a 5- μm sta-

tionary phase. Analyses can be performed at room temperature using a mobile phase of acetonitrile and water. Typical applications of the new column include baseline separation of critical sugars, separation of saccharides from polysaccharides, separation of polysaccharides and maltose/isomaltose ratio determination. The carbohydrate column can be supplied in a 4.5 × 250 mm size.

Circle No. 107 on Reader Service Card.

● Distilled in glass and filtered through 0.2- μm Teflon membranes, Pierce HPLC/spectro grade solvents are intended to have both HPLC and spectrophotometric applications. Label information includes: formula, formula weight, safety data, flash point, first-aid procedures, UV absorbance data, gas chromatography assay, evaporation residue, and water content.

Circle No. 108 on Reader Service Card.

● Two types of guard columns have been introduced by IBM Instruments to prevent liquid chromatography samples with non-eluting materials contaminating HPLC columns. The guard columns are compatible with HPLC instrumentation. One of the columns has normal (silica) pellicular packings while the second has reversed-phase (octadecyl) packings. Each guard column kit contains 4.5 × 50 mm repackable hardware and 6 g of packing material. Replacement packing is available in 10-g units.

Circle No. 109 on Reader Service Card.

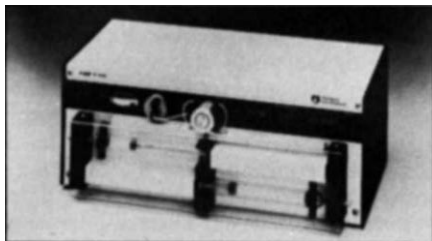
● Beckman Instruments has introduced the model 344M micro-gradient system which is designed to work with 2-mm HPLC columns. The system includes the model 114M pump which features flow ranges of 0.001 to 1.00 ml min⁻¹ or 0.01 to 9.99 ml min⁻¹. A new system organizer incorporates a low column dynamic mixer which is designed to blend solvent combinations at low flow rates.

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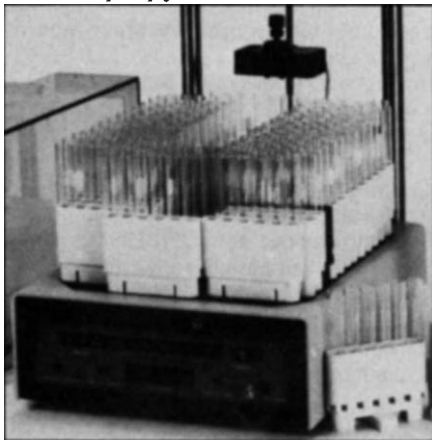


Beckman Instrument's model 344M micro-gradient system

These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.



The P-500 pump from Pharmacia



Isco's Retriever II

Chromatographic accessories

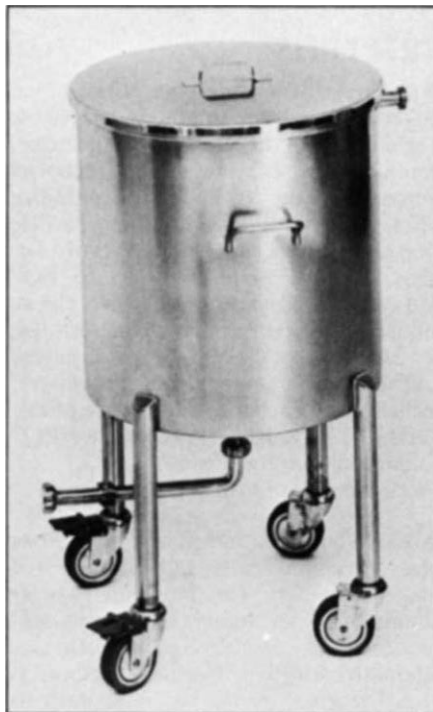
● The new Retriever II linear fraction collector from Isco can handle up to 174 tubes, yet it takes up just a square foot of bench space. Racks are available to hold either 174 of the 12 and 13-mm diameter test tubes, 116 of the 10 to 18-mm tubes, or 42 scintillation vials. The top of the collector can be lifted off. Retriever II can be programmed to collect by time, drop, discharges from a siphon or volumeter, or pulses from a pump.

Circle No. 111 on Reader Service Card.

● Pharmacia Fine Chemicals has introduced a new high precision pump, the P-500. This solvent delivery system is for use when a constant, precise flow is required, as in general liquid chromatography. It is designed for use with Pharmacia's new FPLC high performance chromatography media but is also compatible with traditional separation methods. The pump can be used at atmospheric pressure for columns of Sephadex and Sephacryl. Piston movement is controlled. All materials in contact with the eluent are compatible with aqueous buffers and the borosilicate glass used in the P-500 is impervious to solvents.

Circle No. 112 on Reader Service Card.

● A range of power supplies is now available from Uniscience which produce from 250–4,000 V. It includes the EC300 with the isoelectric focusing end point detector. Also on offer is a family of gradient formers for producing linear, convex, concave and exponential gradients.



Steel tanks from Pharmacia

Two microdialysis models (4 and 24 chambers) are also offered.

Circle No. 113 on Reader Service Card.

● Pharmacia Fine Chemicals has a range of stainless steel tanks that facilitate the preparation, filtration, delivery and collection of buffers and samples used in large scale chromatography. The range is comprised of a buffer preparation tank (capacity 300 litres) fitted with a circulation pump and filter, flat-bottomed buffer tanks (25–300 litres) and conical sample tanks (50–300 litres).

Circle No. 114 on Reader Service Card.

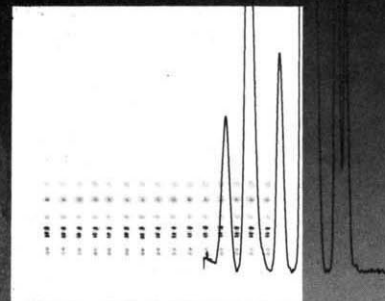
● A dedicated software package has been developed for ion chromatography by Nelson Analytical. This software is offered as part of the standard chromatography software package for Nelson Analytical's 4400 series multi-instrument data systems, which are based on the HP 200 series desktop computers. Data from up to 10 ion chromatographs (20 detectors), or from conventional gas and liquid chromatographs can be processed on the same system simultaneously. Data sampling rates for this system range from one point every 10 seconds to 100 points every second.

Circle No. 115 on Reader Service Card.

● Scientific Glass Engineering is now producing the items required to combine on-column injection with bonded phase capillary columns. These include on-column injectors with special vitreous silica needle syringes, a range of bonded phase columns in vitreous silica and low dead-volume connectors with graphite, vespel or kalrez ferrules.

Circle No. 116 on Reader Service Card.

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Columns and packing materials

● IBM Instruments has added a column for molecular weight analysis to its line of gel permeation chromatography/size exclusion chromatography (GPC/SEC) columns. The new GPC/SEC Scout column is available in a cross-linked divinylbenzene gel. This means that many organic solvents can be used with it, including dimethylformamide. The Scout column simplifies the use of column sets and can be used for molecular weight distribution analysis of polymers with a range of 500 to 4,000,000.

Circle No. 117 on Reader Service Card.

● Pharmacia Fine Chemicals has introduced its Monobeads (see *Nature* 305 95) into prepacked ion exchange columns. An integral part of their fast protein liquid chromatography (FPLC) system, Monobeads represent a new generation of pressure and pH stable chromatographic absorbents. They have been designed for rapid protein, peptide and polynucleotide separation. The Monobeads are available in 3 dimensions — Mono Q for anion exchange, Mono S for cation exchange and Mono P, a chromatofocusing matrix which separates proteins according to their isoelectric points. Their monodispersity allows high efficiency packing and medium pressure operation. They feature a particle size distribution of $9.8 \mu\text{m} + 2\%$. The prepacked columns are made of borosilicate glass, which enables the gel bed to be seen.

Circle No. 118 on Reader Service Card.

● A series of wide-pore silica-based materials has been produced by HPLC Technology for the separation of proteins, peptides and enzymes. The TechoGel range comprises 200 and 300 Å pore-size silicas, in both 5- μm and 10- μm particle sizes. The TechoGel range consists of a GP phase for gel permeation up to MW 250,000; ion exchange, which covers weak anions, weak cations and strong cations; reverse phase — C4, C6, C8, C18, phenyl and diphenyl and preparative phases, including ion exchange and reverse phase. TechoGel is available in prepacked HPLC columns or as loose media.

Circle No. 119 on Reader Service Card.

● Du Pont has introduced a Zorbax phenyl column for HPLC with reversed phase selectivity for moderately polar compounds, including many pharmaceuticals. The new phenyl column offers an alternative selectivity to other Zorbax alkyl group reversed phase packings, such as ODS, C8 and TMS. This means that chromatograms of two photoreactive chemicals (cyclohexylacrylate and benzophenone) eluted from Zorbax phenyl and C8 columns under identical conditions will show reversed elution order. The Zorbax phenyl column also gives a small shoulder on the dimethoxyacetophenone peak. The phenyl column contains 5- μm Zorbax silica packing modified with a dimethylaryl silane, using procedures to maximize monolayer surface coverage. Post capping completes surface coverage. The columns have a 4.6-mm internal diameter and are available in 15 and 25 cm lengths. Performance characteristics described by typical theoretical plates (N) are 9,000 for the 15-cm column and 15,000 for the 25-cm column. The compound used in the characterization was toluene.

Circle No. 120 on Reader Service Card.

● The new liquid chromatography chambers from Revco have four temperature controls, display temperature in all four quadrants continuously and have an alarm system that tests itself automatically every 24 h. They also have computer connections for collecting, storing and monitoring temperature data automatically. The chambers also have an optional auto-dialer alarm system whereby they will call any of 10 numbers, a 240-day electronic temperature recorder and a remote audio alarm.

Circle No. 121 on Reader Service Card.

● Two new chiral columns are now being produced by J.T. Baker Chemicals that are designed to separate racemic mixtures and stereoisomers. Two types of column have been produced; the DNBPG ionically bound to 5- μm aminopropyl silica (60-Å) for lipophilic samples and a covalently-bound DNBPG which is suitable for pharmaceutical applications.

Circle No. 122 on Reader Service Card.

● J.T. Baker has introduced a range of HPLC columns containing Bakerbond materials bonded to 5- μm spherical silica gel with a pore diameter of 60 Å. The range includes octadecyl (C18), octyl (C8), quaternary amine, aliphatic sulphonic acid, diol, aliphatic carboxyl, diphenyl and cyano. The columns are available in the standard size of 4.6 mm (internal diameter) $\times$ 25 cm with SSI fittings. In addition a number of wide-pore columns has been introduced for the separation and purification of proteins, peptides and oligonucleotides. The wide-pore columns have a pore diameter of 330 Å. The range of columns includes octadecyl, octyl, diphenyl, cyano and polyethyleneimine. The Bakerbond bonded phases are also available separately with particle sizes of 5 μm (spherical), 10 μm (irregular) and 40 μm (irregular).

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Electrophoresis

● The Dionex IonPac membrane reactor uses a new method of reagent introduction which aids in the determination of metals, sequestering agents, silicates and amino acids. The membrane reactor is for use with the Dionex post column reactor and a series 2000i ion chromatograph. The membrane reactor's fibre membrane is permeable to a variety of reagents. During operation, pressurized reagent from the pulseless post column reactor is introduced into the column effluent stream along the length of the fibre membrane. This means that reagents are added at a pulse-free flow-dependent rate. The volume of reagent introduced in this method is minimal, reducing analyte dilution effects. Flow-dependent noise and dilution effects are also reduced.

Circle No. 124 on Reader Service Card.

● A casting tray is being marketed by Bio-Rad that can be used to produce ultra-thin gels for thin layer electrofocusing. The tray allows 0.4-mm polyacrylamide gels to be cast on glass plates or, when used in conjunction with Bio-Rad's new gel support film for polyacrylamide, allows 0.2-mm polyacrylamide gels to be produced.

Circle No. 125 on Reader Service Card.

● The Gelman electrophoresis system, which uses cellulose acetate membranes as support medium, is based upon the established semi-micro II electrophoresis chamber. This chamber has been designed to reduce running costs. It requires 200 ml of buffer to effect separation of up to 96 samples, including standards and controls. Up to 24 samples may be separated simultaneously. Sample application is by either a four- or an eight-place push button

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